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Russell George Rogers III

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## ACCEPTANCE

This dissertation, ANTIOXIDANT THERAPY ATTENUATES POST-INFARCT CARDIAC REMODELING BY DRIVING EXPRESSION OF KRÜPPEL-LIKE FACTOR 15, by RUSSELL G. ROGERS III, was prepared under the direction of the candidate's Dissertation Advisory Committee. It is accepted by the committee members in partial fulfillment of the requirements for the degree, Doctor of Philosophy, in the College of Education and Human Development, Georgia State University.

The Dissertation Advisory Committee and the student's Department Chairperson, as representatives of the faculty, certify that this dissertation has met all standards of excellence and scholarship as determined by the faculty.

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- 2) Baumann, C.W., **Rogers, R.G.**, Lees, S.J., & Otis, J.S. (2014) Muscular Strength is Unaffected by Short-Term Resveratrol Supplementation in Aged Mouse Muscle. *International Journal of Clinical and Experimental Physiology*, 1, 253-257.
- 3) Baumann, C.W., **Rogers, R.G.**, Gahlot, N., & Ingalls C.P. (2014) Eccentric Contractions Disrupt FKBP12 Content in Mouse Skeletal Muscle. *Physiological Reports*, 2 (7), e12081.

### *Published Abstracts*

- 1) **Rogers, R.G.**, Baumann, C.W., & Otis, J.S. (2015). Recovery of Skeletal Muscle Function is Not Augmented by Acute Resveratrol Supplementation after Injury. *Medicine and Science in Sports and Exercise*, 47, 960-961.
- 2) Baumann, C.W., **Rogers, R.G.**, & Otis, J.S. (2015). Functional Deficits are Not Attenuated by Increasing Hsp70 Content Prior to Trauma-induced Injury. *Medicine and Science in Sports and Exercise*, 47, 505-510.
- 3) **Rogers, R.G.**, Baumann, C.W., Santamore, W., Gorman, J.H., Gorman, R.C., & Ingalls, C.P. (2014). A Biocompatible Tissue Filler Attenuates Junctophilin 2 Loss after A Myocardial Infarction In Sheep Heart. *Medicine and Science in Sports and Exercise*, 46, 660-661.
- 4) Baumann, C.W., **Rogers, R.G.**, Gahlot, N., & Ingalls, C.P. (2014). Loss of FKBP12 is Associated with Early Strength Deficits after Contraction Induced Skeletal Muscle Injury. *Medicine and Science in Sports and Exercise*, 46, 992.

### *Poster Presentations at National and International Conferences*

- 1) **Rogers, R.G.**, & Otis, J.S. Antioxidant Therapy Attenuates Post-Infarct Left Ventricular Remodeling by Driving Expression of the Transcriptional Repressor Krüppel-Like Factor 15. *Experimental Biology*. San Diego, CA, April 2016.
- 2) **Rogers, R.G.**, Baumann, C.W., & Otis, J.S. Recovery of Skeletal Muscle Function is Not Augmented by Acute Resveratrol Supplementation after Injury. *American College of Sports Medicine Annual Meeting*. San Diego, CA, May 2015.
- 3) **Rogers, R.G.**, Baumann, C.W., & Otis, J.S. Recovery of Skeletal Muscle Function is Not Augmented by Acute Resveratrol Supplementation after Injury. *American College of Sports Medicine Southeast Regional Chapter Annual Meeting*. Jacksonville, FL, February 2015.
- 4) **Rogers, R.G.**, Baumann, C.W., Santamore, W., Gorman, J.H., Gorman, R.C., & Ingalls, C.P. A Biocompatible Tissue Filler Attenuates Junctophilin 2 Loss after A Myocardial Infarction In Sheep Heart. *American College of Sports Medicine Annual Meeting*. Orlando, FL, May 2014.

ANTIOXIDANT THERAPY ATTENUATES POST-INFARCT CARDIAC REMODELING  
BY DRIVING EXPRESSION OF KRÜPPEL-LIKE FACTOR 15

by

RUSSELL G. ROGERS, III

Under the Direction of Jeffrey S. Otis, Ph.D.

## ABSTRACT

**Background:** Myocardial infarction (MI) results in severe biochemical, physiological, and cellular changes that lead to alterations in the structure and function of the myocardium. Oxidative stress potentiates this remodeling response and is associated with progressive worsening of cardiac function. Accordingly, we used a powerful antioxidant-based therapeutic strategy to improve cardiac health and study redox-dependent signaling. **Methods:** MI was surgically induced in rats by ligating the left anterior descending coronary artery. Subgroups of MI rats received resveratrol (i.p., 10 mg/kg/day for 28 days beginning immediately post-MI). Cardiac histology and biochemical analyses of genes and proteins implicated in cardiac fibrosis, hypertrophy, and apoptosis, and redox-dependent signaling were analyzed. **Results:** As expected, MI resulted in profound structural changes to the myocardium. Further, we observed a sharp reduction in nuclear factor-erythroid 2-related factor 2 (Nrf2) and Krüppel-like factor 15 (KLF15), factors that are responsible for maintaining the endogenous antioxidant capacity and regulating cardiac gene expression, respectively. It is likely that disruption of normal KLF15 signaling permitted the expression of several cardiac genes associated with progressive cardiac remodeling. Importantly, daily treatment with resveratrol ameliorated cardiac remodeling, improved redox state, restored Nrf2 expression, and up-regulated KLF15 expression. Further, induction of KLF15 signaling following resveratrol treatment is associated with attenuated expression of several genes implicated in cardiac remodeling. **Conclusions:** Chronic oxidative stress potentiates cardiac remodeling post-infarct, in part, by suppressing Nrf2 and KLF15 expression. Importantly, we demonstrate that normal KLF15 signaling may be rescued with an antioxidant-based therapy, which may be an attractive therapeutic target to support cardiac health post-MI.

**INDEX WORDS:** Cardiac remodeling, KLF15, Myocardial infarction, Oxidative stress



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BY DRIVING EXPRESSION OF KRÜPPEL-LIKE FACTOR 15

by

RUSSELL G. ROGERS, III

A Dissertation

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Doctor of Philosophy

in

Kinesiology (Exercise Physiology: Muscle Biology)

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Department of Kinesiology and Health

in

The College of Education and Human Development

Georgia State University

Atlanta, GA  
2016

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## **DEDICATION**

I would like to dedicate this dissertation, in partial fulfillment of the Doctor of Philosophy degree, to my family, friends, and colleagues that provided support during my collegiate career – thank you.

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## ABBREVIATIONS

ANP	Atrial natriuretic peptide
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BNP	B-type natriuretic peptide
CTGF	Connective tissue growth factor
Cu/Zn-SOD1	Copper-zinc superoxide dismutase
CVD	Cardiovascular disease
DNA	Deoxyribonucleic acid
Ec-SOD3	Extracellular superoxide dismutase
GATA4	GATA binding protein 4
GPx	Glutathione peroxidase
GSH	Glutathione (reduced form)
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
IGF-1	Insulin-like growth factor-1
IL-1 $\beta$	Interleukin-1beta
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
KChIP2	K <sub>v</sub> channel-interacting protein 2
KLF	Krüppel-like factor family
LAD	Left anterior descending coronary artery
Mef2	Myocyte enhancer factor 2
MI	Myocardial infarction
Mn-SOD2	Manganese superoxide dismutase
mPTP	Mitochondrial permeability transition pore
NAD <sup>+</sup>	Nicotinic adenine dinucleotide (oxidized form)
NADH	Nicotinic adenine dinucleotide (reduced form)
NADPH	Nicotinic adenine dinucleotide phosphate (reduced form)
NF- $\kappa$ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
Nox2	Nicotinic adenine dinucleotide phosphate oxidase 2
Nox4	Nicotinic adenine dinucleotide phosphate oxidase 4
Nrf2	Nuclear factor-erythroid 2-related factor
O <sub>2</sub> <sup>-</sup>	Superoxide
OH <sup>-</sup>	Hydroxyl radical
P/CAF	P300/CREB-binding protein-associated factor
PGC-1	Peroxisome proliferator-activated receptor-gamma co-activator-1
PPAR- $\alpha$	Peroxisome proliferator-activated receptor-alpha
ROS	Reactive oxygen species
SERCA	Sarcoplasmic-endoplasmic reticulum calcium ATPase
Smad3	Mothers against decapentaplegic homolog 3
SOD	Superoxide dismutase
TGF- $\beta$	Transforming growth factor-beta
TLR	Toll-like receptor
TNF- $\alpha$	Tumor necrosis factor-alpha
TRAIL	TNF-related apoptosis-inducing ligand

## CHAPTER ONE

### MYOCARDIAL INFARCTION AND CARDIAC REMODELING

#### **Introduction**

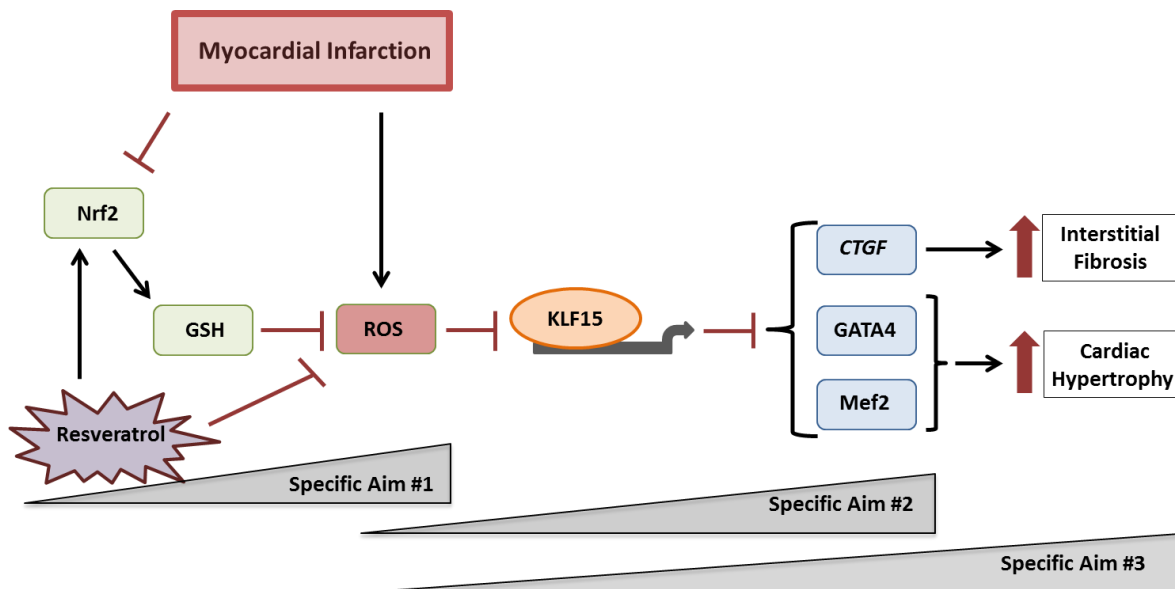
The high prevalence of cardiovascular disease (CVD) in the Western world is responsible for approximately thirty-three percent of all-cause mortality in the United States. In addition, each year nearly 1.5 million Americans experience a myocardial infarction [1, 2], which directly contributes to mortality or secondary heart failure. The Centers for Disease Control and Prevention estimate that 5.1 million people have been diagnosed with heart failure and approximately half die within 5 years of diagnosis. Clearly, continued research investigating the molecular and cellular mechanisms underlying the pathogenesis of CVD to develop effective treatment options is warranted.

Importantly, myocardial infarction has been associated with a series of pathological molecular and cellular changes that result in remodeling of the myocardium. The pathologic myocardium is associated with cardiac dysfunction as it progresses to secondary heart failure. Moreover, several lines of literature implicate oxidative stress as a primary feature of cardiac remodeling following clinical and experimental myocardial infarction. Genetic studies that employ either knockout of reactive oxygen species producing enzymes or overexpression of antioxidant defense enzymes demonstrate remarkable protection of the myocardium to pathological remodeling. Importantly, treatment with antioxidants has also proven beneficial to attenuate cardiac remodeling following myocardial infarction. Together, these studies establish a role for oxidative

stress in the promotion of a pathological cardiac phenotype. However, the underlying mechanisms that drive oxidative stress-dependent remodeling following myocardial infarction have not been fully elucidated.

Further, interstitial fibrosis and cardiac hypertrophy are cardinal features of the remodeling myocardium in response to pathological stress. These processes appear to be under strict control of connective tissue growth factor (CTGF), myocyte enhancer factor 2 (Mef2), and GATA binding protein 4 (GATA4), which are robustly up-regulated in response to pressure overload. Importantly, the transcriptional repressor Krüppel-like factor 15 (KLF15) negatively regulates the transcription of CTGF and transcriptional activity of Mef2 and GATA4. In response to *in vivo* pressure overload and *in vitro* oxidative stress, KLF15 is dramatically down-regulated which alleviates its inhibitory effects and thus, permits expression of pro-fibrotic and pro-hypertrophic genes.

While antioxidant treatment has proven beneficial in attenuating cardiac remodeling following myocardial infarction, the redox-sensitive mechanisms responsible for its efficacy remain to be fully identified. To that end, no study to date has identified the expression of KLF15 in the myocardium following infarction. Here we attempt to identify the expression profile of KLF15 and its sensitivity to the redox state in the myocardium following infarction. Importantly, we hypothesize that treatment with resveratrol, a powerful antioxidant, will induce KLF15 expression to repress cardiac gene expression associated with remodeling post-MI. For clarity, we have provided the hypotheses and specific aims for the proposed dissertation project in Figure 1.



**Figure 1.** Hypotheses and specific aims wherein myocardial infarction induces oxidative stress (SA 1), which down-regulates KLF15 and permits the expression of fibrogenic and hypertrophic genes (SA 2). Expression of these genes leads to overt structural remodeling of the myocardium (SA 3). Treatment with resveratrol induces Nrf2 signaling to restore GSH and alleviate oxidative stress, thereby driving KLF15 signaling and attenuate cardiac remodeling. Connective Tissue Growth Factor (CTGF), GATA Binding Protein 4 (GATA4), Glutathione (GSH), Krüppel-Like Factor 15 (KLF15), Myocyte Enhancer Factor 2 (Mef2), Nuclear Factor erythroid-derived 2-like factor 2 (Nrf2), Reactive Oxygen Species (ROS), Specific Aim (SA).

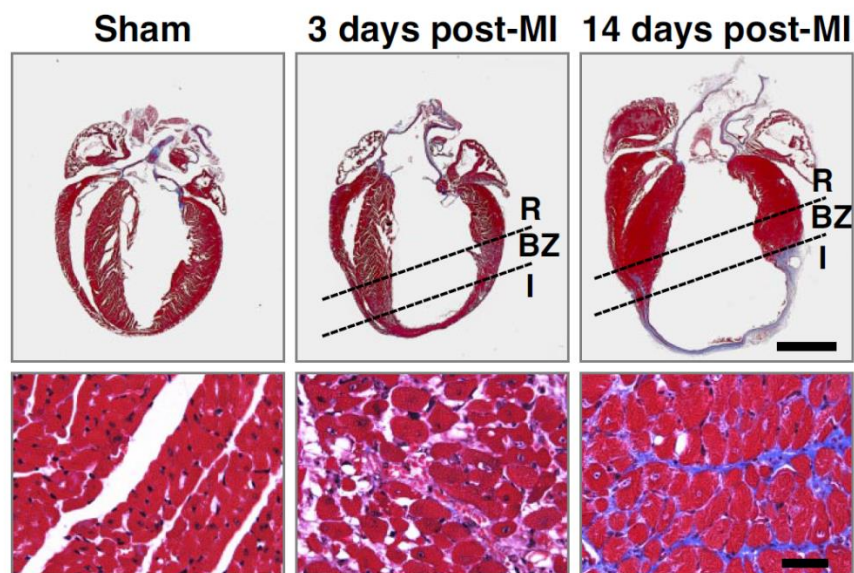
### Characteristics of Myocardial Infarction

Myocardial infarction can be characterized by acute and chronic structural and functional alterations that occur within minutes and manifest as overt structural remodeling weeks to months after the initial ischemic insult. Changes in myocyte number, size, and shape manifest as overt structural remodeling of the myocardium and are closely associated with functional deterioration. The direct relationship between the structure and function of the myocardium underscores the essential requirement to prevent pathological cardiac remodeling and to preserve cardiac function.

### *Histopathology of Myocardial Infarction*

The myocardium undergoes several morphological changes within hours after the initial ischemic insult, which persists for several weeks to months following myocardial infarction. During the infarction, cardiac myocytes in the ischemic zone experience necrotic cell death characterized by cell swelling and disruption of the plasma and organelle membranes. Further, cardiac myocytes in the non-infarcted region may undergo apoptotic cell death characterized by cell shrinking and condensed nuclei [3, 4].

Additionally, excessive destruction of the extracellular matrix may destabilize the collagen struts, which normally provide cellular stabilization to cardiac myocytes. As such, cardiac myocytes in the border zone are likely to slip and potentially contribute to expansion of the infarct and ventricular wall thinning. Cell loss and extracellular matrix destruction increase myocardial wall stress. To that end, cardiac myocytes that survive hypertrophy and the myocardium thickens early in the remodeling process. Over time, chronic stress induces a shift from a thicker to a longer myocyte, which coupled with infarct expansion manifest as increased ventricular cavity dimension. In parallel, chronic stress likely contributes to interstitial collagen deposition and subsequent fibrosis (Figure 2).



**Figure 2.** Photomicrographs from mouse heart sections. Three days post-MI, the infarct zone (I) begins to form from the resultant loss of cardiomyocytes and scar tissue formation. The region adjacent to the infarct zone, termed the border zone (BZ), distinguished by the appearance of interstitial collagen deposition and cardiomyocyte hypertrophy appears. Adjacent to the border zone, the remote myocardium (R), experiences a lesser degree of stress than the border zone, and thus a lesser degree of remodeling. These structural derangements are augmented at 14 days post-MI, where there is apparent increase in the ventricular chamber radius. Further, the infarct zone continues to thin and expand with a dense collagen framework. In parallel, cardiomyocytes in the border zone experience hypertrophy concomitant with interstitial fibrosis [5].

### *Functional Evidence of Myocardial Infarction*

Ischemic cardiomyopathy resulting from a myocardial infarction is commonly associated with systolic dysfunction—a hallmark of heart failure. Intrinsic and structural alterations manifest as significant deterioration to many hemodynamic parameters that define cardiac performance. The underlying mechanisms responsible for systolic dysfunction include: post-translational modification of myofilament proteins that result in force development depression and decreased calcium sensitivity of the contractile apparatus, dysfunctional calcium handling, altered ion channel function, mitochondrial and metabolic abnormalities, and pathological structural remodeling [6].

Systolic function can be assessed with data from pressure-volume loops such as stroke volume, ejection fraction, stroke work, end-systolic pressure-volume relation, and end-diastolic pressure-volume relation. Relative narrowing of the pressure-volume loop accompanied by a rightward shift is reflective of reduced stroke volume, increased end-diastolic volume, and subsequently reduced ejection fraction indicative of an infarcted and failing heart [6, 7]. For example, several studies report significant depression of systolic function following experimental myocardial infarction induced by LAD ligation [8-11]. Four weeks after myocardial infarction ventricular fractional shortening [8-11] and left ventricular ejection fraction [8, 11] are dramatically reduced. In parallel, these functional deficits were associated with pathological cardiac remodeling. Importantly, animals that receive antioxidant interventions show remarkable improvement in both indices of systolic function and pathological remodeling [8-11].

### **Molecular and Cellular Events during Acute Myocardial Infarction**

The myocardium undergoes several molecular and cellular events starting with the initial ischemic insult leading to cell death. The ensuing inflammatory response then clears the myocardium of dead cells and matrix debris, and prepares the myocardium for structural remodeling.

#### *Etiology of Myocardial Ischemia*

Embolization or passage of an embolus (i.e., coronary plaque) within the bloodstream can occlude one or more major coronary arteries and deprive the myocardium of sufficient oxygen [12]. Plaque embolization is due to damage of the underlying vascular endothelium that results in formation of platelet plugs and thrombi [13]. Rupture of the plaque exposes thrombogenic ele-

ments in the atherosclerotic lesion to the moving column of blood. Traveling thrombogenic elements trigger platelet aggregation and thrombus formation and result in occlusion of the diseased vessel [13]. To that end, total occlusion of a major coronary artery such as the left anterior descending coronary artery results in the entire thickness (i.e., the subepicardium and subendocardium) of the left ventricle to become ischemic, known as *transmural ischemia* [13]. Alternatively, the partial occlusion of major arteries or significant collateral vessels results in fractional ischemic areas of the ventricular wall. When this occurs, the under-perfused subendocardium becomes more susceptible to ischemic shock [13].

#### *Anoxic Intracellular Pathophysiology*

Intracellular oxygen tension within the affected myocardium falls to nearly zero within a minute of complete cessation of blood flow [13]. As a result, reliance on oxidative phosphorylation to generate adenosine triphosphate (ATP) is significantly reduced, and ATP generation is relegated to anaerobic glycolysis [13]. Unfortunately, anaerobic energy production can only proceed in the presence of sufficient cytosolic nicotinamide adenine dinucleotide ( $\text{NAD}^+$ ). Since the reduced form of  $\text{NAD}^+$  ( $\text{NADH} + \text{H}^+$ ) can only be oxidized via oxidative phosphorylation, the concentration of  $\text{NAD}^+$  quickly falls and anaerobic glycolysis ceases. ATP consuming enzymes such as myosin ATPase, sarcoplasmic-endoplasmic reticulum calcium ATPase (SERCA), and membrane bound sodium-potassium ATPase pump consume the remaining ATP. The immediate and cumulative result is abnormal cellular function, energy depletion, and eventual cell death.



### *Cellular Death Pathways*

Myocardial cell loss during acute myocardial infarction is a consequence of necrosis, apoptosis, and autophagy [14-16]. Each death pathway has distinguishing features, are not mutually exclusive [14], and their contribution to cell loss is likely specific to the cellular and extracellular environment. For example, different pathways may contribute variably between models of myocardial infarction (e.g., permanent artery occlusion vs. ischemia/reperfusion) or due to the state of cellular energy demands.

Necrosis is an unregulated, irreversible response to sustained ischemia and is the primary driving force of cell loss during myocardial infarction [14]. Due to cellular energy failure, sodium and calcium ions accumulate in the cytoplasm and cause cell swelling, degeneration of organelles, loss of membrane integrity, and dissolution of the cell [17]. Upon restoration of blood flow, necrosis continues in the infarct zone, but it may be mediated through different processes. Until recently, reperfusion-induced necrosis was considered a passive event referred to as “coagulation necrosis” where the architecture of necrotic cells remains preserved for a couple days [14, 18]. However, it is now clear that cellular signaling pathways are capable of regulating necrosis [3, 4]. Recently, this form of necrosis has been termed “programmed necrosis” and can be initiated by tumor necrosis factor-alpha (TNF- $\alpha$ ). Primary features of programmed necrosis are swelling of the mitochondrial matrix, dispersion of the mitochondrial membrane potential, ATP depletion, and opening of the mitochondrial permeability transition pore (mPTP). Activation of caspases is not a feature of programmed necrosis; however, it may occur in parallel if matrix swelling causes rupture of the outer mitochondrial membrane before sufficient ATP is depleted. In this context, pro-apoptotic factors such as cytochrome *c* are released into the cytosol and potentially activate caspases [14].

Cell death mediated through apoptosis requires energy to activate caspases. Therefore, it is likely that less energy-compromised cells in the border zone are more likely to die by apoptosis and contribute to infarct expansion [14]. However, during reperfusion (when cellular energy level is restored) apoptosis may occur in the infarct zone [19] and is likely driven by oxidative stress in a graded manner [20]. For example, cardiac myocytes closest to the capillaries receive the highest level of oxidative stress and therefore, may contribute to independent initiation of necrosis and apoptosis during reperfusion.

Activation of caspases is a hallmark biochemical feature of apoptosis and are activated by two major pathways [14]. First, the extrinsic pathway is initiated as a cellular response to inflammation. Plasma membrane receptors become activated by pro-inflammatory ligands such as Fas, TNF- $\alpha$ , and TNF-related apoptosis-inducing ligand (TRAIL) [14]. These ligands bind death domain-containing receptors to form a death-inducing signaling complex, which initiates proteolytic cleavage of pro-caspases and subsequently activates effector caspases [14]. Alternatively, the intrinsic pathway requires the permeabilization of the outer mitochondrial membrane to release mitochondrial pro-apoptotic factors such as cytochrome *c* [14]. Importantly, elevated cytosolic calcium ions and reactive oxygen species (ROS) have been implicated in activating the intrinsic pathway [14]. To that end, the effector caspase, caspase-3 digests cellular proteins and macromolecules, degrades DNA, and leads to cell death [14, 21].

Normally, autophagy provides a “housekeeping” function that degrades damaged organelles and macromolecules in response to stress, and promotes cell survival [22]. Alternatively, during persistent stress autophagy can initiate a cellular death response [23]. In support of these roles, autophagy may protect the myocardium and limit the size of the infarct during ischemia

[16, 24]. Alternatively, reperfusion-induced autophagy activation promotes expansion of the infarct zone [25]. It is now clear that different pathways are responsible for the diverse roles of autophagy to promote cell survival and cell death [16, 26]

### *Inflammatory Response*

Disruption of plasma membrane integrity is a common feature of necrosis and results in the release of intracellular contents into the extracellular and systemic compartments that initiate a robust inflammatory response [27]. Cell surface receptors bind endogenous ligands released from necrotic cells and activate inflammatory pathways such as Toll-like receptor (TLR)-mediated pathways, the complement cascade, and the nuclear factor (NF)- $\kappa$ B cascade. To that end, NF- $\kappa$ B plays an essential role in the induction of pro-inflammatory mediators such as TNF- $\alpha$ , interleukin 1-beta (IL-1 $\beta$ ), IL-6, and ROS. Importantly, NF- $\kappa$ B can be reciprocally activated by TNF- $\alpha$  and ROS [27].

Moreover, ROS promote leukocyte chemotaxis by complement activation, and up-regulation of adhesion molecules and chemokines [28]. The first inflammatory cell type in the peripheral circulation to increase in numbers after the ischemic insult are neutrophils [29]. Their primary role is to release large amounts of ROS through a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent respiratory burst, which functions to degrade damaged particles [30]. Importantly, ROS released from inflammatory infiltrates and dying cells may directly injure healthy cardiac myocytes and vascular cells and contribute to myocardial damage [28].

As such, clearance of dead cells and debris, and inhibition of cytokine and chemokine synthesis is crucial for the repair process. Further, optimal healing requires mechanisms that inhibit cytokine and chemokine synthesis to suppress continuous leukocyte recruitment and injury.

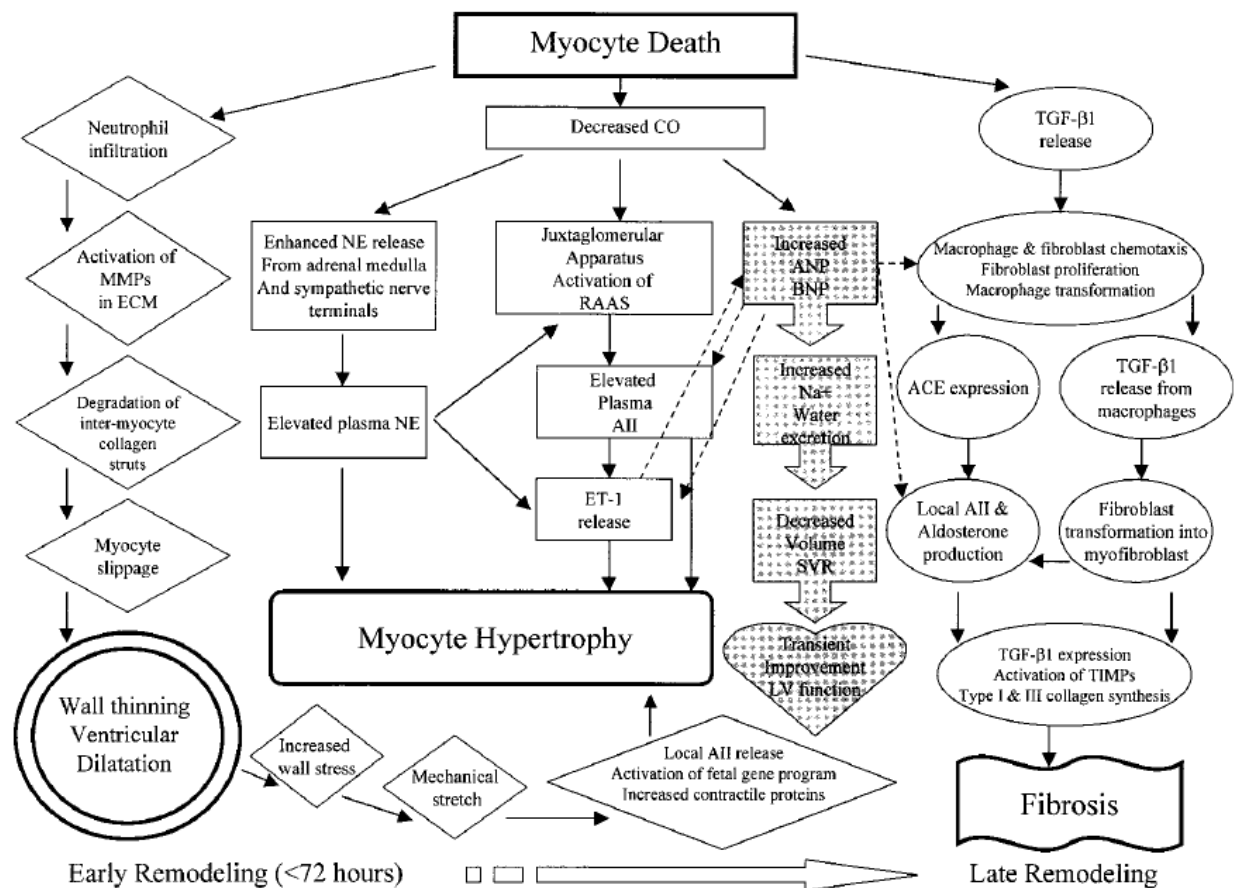
To that end, macrophage ingestion of apoptotic cells including neutrophils and cardiac myocytes results in powerful anti-inflammatory and immunosuppressive effects that transition to fibrous tissue deposition to stabilize the damaged myocardium [27].

Transforming growth factor- $\beta$  (TGF- $\beta$ ) is a key mediator in the transition from inflammation to fibrotic tissue deposition [31]. Moreover, TGF- $\beta$  suppresses cytokine and chemokine expression by stimulated mononuclear and endothelial cells [27]. Importantly, TGF- $\beta$  inhibits proliferation of most cells, modulates fibroblast behavior, stimulates synthesis of various extracellular matrix proteins [32], and suppresses matrix degradation [33]. The fibroblast conversion to a myofibroblast phenotype is a primary feature of infarct scar formation and is characterized by increased expression of  $\alpha$ -smooth muscle actin, cell proliferation, and extracellular matrix protein synthesis [34].

Myofibroblasts are not normally present in high numbers in the healthy myocardium. However, in response to mechanical stress and hormones released by inflammatory and resident cells, myofibroblasts migrate to damaged tissue [35]. Three days following infarction, they are the predominant cell type in the infarct zone [35]. Importantly, myofibroblasts serve two primary functions in the infarcted myocardium: (1) provide mechanical strength to the scar by secreting new extracellular matrix proteins and (2) synthesize factors that regulate the inflammatory and fibrogenic responses [35]. Initially, myofibroblasts secrete a specific set of matrix proteins to form a provisional scar to provide temporary stabilization of the ventricular myocardium. Eventually, the provisional scar is replaced with a more advanced scar containing a stable collagen network [34, 36].

## Post-Infarct Cardiac Remodeling

The damaged myocardium undergoes profound changes in the ventricular architecture and geometry, referred to as “cardiac remodeling” [27]. Several molecular and cellular alterations are associated with cardiac remodeling that affects both the infarct zone and non-infarct segments of the ventricle. Over time, continued cardiac remodeling can become pathological in nature and manifest as apoptosis of viable cardiac myocytes, interstitial fibrosis, cardiac hypertrophy, dilation of the left ventricle, and worsened cardiac function [27, 37, 38]. Figure 3 provides an overview of the factors that contribute to pathological cardiac remodeling.



**Figure 3.** Flow chart representing the many factors involved in the pathophysiology of ventricular remodeling [39]. Angiotensin II (AII), Angiotensin Converting Enzyme (ACE), Atrial Natriuretic Peptide (ANP), B-Type Natriuretic Peptide (BNP), Cardiac Output (CO), Extracellular Matrix (ECM), Endothelin-1 (ET-1), Matrix Metalloproteinase (MMP), Norepinephrine (NE), Renin-Angiotensin-Aldosterone System (RAAS), Systemic Vascular Resistance (SVR), Transforming Growth Factor-Beta1 (TGF- $\beta$ 1).

Further, loss of viable cardiac myocytes is an important mechanism in the development of pathological cardiac remodeling [40]. Shear wall stress imposed on cardiac myocytes lining the infarct scar induce oxidative stress and activate a second inflammatory wave within these cells. Experimental evidence document the expression of both TNF- $\alpha$  [41] and inducible nitric oxide synthase (iNOS) [42] in cardiac myocytes bordering the infarct scar. Importantly, oxidative and nitrosative stress lead to apoptosis in cardiac myocytes adjoining the infarct scar, which, in conjunction with cell slippage, result in expansion of the infarct zone [43]. To that end, fibrous tissue replaces dead cardiac myocytes and contributes to extension of the infarct scar [44].

The adjacent non-infarcted region defined by its proximity to the infarct zone is commonly referred to as the “border zone.” Surgically implanted sonomicrometers demonstrate that the non-infarcted myocardium in the border zone can be progressively recruited into the mature scar. In other words, there is an increased proportion of the left ventricular wall composed of scar tissue and decreased proportion composed of viable cardiac myocytes during the remodeling response [45]. Therefore, the border zone may not remain in a fixed position after formation of the mature scar in the infarct zone.

In addition to infarct scar extension, oxidative stress promotes interstitial collagen deposition in the non-infarcted myocardium and contributes to restructuring of the myocardium [44]. Further, the non-infarcted myocardium lying beyond the border zone may hypertrophy in re-

response to a workload increase imposed on these cardiac myocytes. The magnitude of the hypertrophic response is dependent on several factors including: size of the initial infarct, type of infarct, location of the infarct, type of reperfusion, degree of infarct extension, ventricular preload and afterload, and the state of inflammatory activation. Thus, it seems reasonable to presume that the onset of the hypertrophic response will vary depending on the sum total of these and perhaps additional factors such as oxidative stress [44].

In later stages of cardiac remodeling, the myocardium enters a state primarily driven by chronic volume overload, which induces a characteristic dilated myocardium, in part, by lengthening of cardiac myocytes [44]. In parallel, increased ventricular volume concomitant with decreased subendocardial perfusion drive elevated wall stress and result in depressed ventricular ejection fraction [44, 46]. Importantly, ventricular dilation is associated with development of heart failure, ventricular arrhythmias [47], and has been used to predict mortality [48].

Progression to heart failure secondary to uncomplicated myocardial infarction can be defined in terms of the function, shape, and size of the left ventricle [49]. For example, infarct size correlates well with both end-systolic volume and ejection fraction [50]. Further, infarct imaging demonstrates a direct relationship between infarct scar size, both ventricular volumes, and ejection fraction [51]. Given that elevated end-systolic volume can be predicted from infarct size and end-systolic volume is a major determinant of mortality following myocardial infarction [48], extensive cardiac remodeling has been used as a surrogate endpoint for use in heart failure trials [52, 53]. Importantly, cardiac remodeling should be considered a primary target to prevent secondary heart failure following myocardial infarction [54, 55]. Therefore, a better understanding of the mechanisms responsible for pathological cardiac remodeling to develop effective therapeutic strategies is warranted.

### *Oxidative Stress in the Myocardium*

Oxidative stress occurs when the production of reactive oxygen species such as superoxide ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), and hydroxyl radical ( $OH^\cdot$ ) exceeds the cellular antioxidant defense capacity and promotes their rapid accumulation [56]. Importantly, the unpaired electron is an unstable free radical that will react with organic molecules such as proteins, lipids, and nucleic acids and lead to disruption of cellular function [6]. ROS production occurs through electron leak from mitochondria during oxidative phosphorylation and through activation of cellular enzymes such as NADPH oxidase, xanthine oxidase, and NOS [57].

In both the infarcted and non-infarcted myocardium, NADPH oxidases are a major source of oxidant production [58, 59]. However, the cellular source may differ between the two areas. For example, NADPH oxidase expression robustly increases in the infarct zone [60, 61] and leukocytes are the primary cell type to express the enzyme [57], while cardiomyocytes are more likely to express the enzyme in non-infarcted myocardium [62, 63]. Further, pathological stimuli such as  $TNF-\alpha$ , angiotensin II, norepinephrine, and mechanical stretch increase the activity of NADPH oxidase enzymes [9], which occur as a result of an ischemic insult. Therefore, an effective oxidant scavenging system to combat oxidant production following a myocardial infarction is required to alleviate the accumulation of ROS.

Nuclear factor-erythroid 2-related factor 2 (Nrf2) is a highly conserved transcription factor that induces transcriptional activation of several anti-oxidant and phase II detoxifying enzymes that harbor the antioxidant response element (ARE) in their promoter region [64]. Enzymes under the transcriptional regulation of Nrf2 may include: NADPH dehydrogenase, superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione *S*-transferase,  $\gamma$ -glutamate-cysteine ligase, heme oxygenase-1, and catalase [64, 65].



Importantly, glutathione (GSH) is a major product of several Nrf2-regulated genes and is the most abundant intracellular non-enzymatic free thiol that functions in antioxidant defense. GSH can reduce  $H_2O_2$  and lipid peroxide through a GPx catalyzed reaction and detoxify electrophiles spontaneously or through a glutathione *S*-transferase catalyzed reaction [66]. Therefore, adequate cellular GSH concentration is vital for normal cell function [67], whereas a reduced concentration of GSH may promote accumulation of ROS and subsequent oxidative stress.

Further, mitochondrial enzymes manganese superoxide dismutase (Mn-SOD2) and GPx appear to be the most important in controlling myocardial levels of  $O_2^-$  and  $H_2O_2$ . Approximately 90% of the SOD activity in cardiac myocytes is attributable to Mn-SOD2 [6]. For example, a study by Li et al [68] highlighted the strict requirement of Mn-SOD2 in the regulation of oxidative stress in the myocardium. Using homozygous knockout mice, they demonstrated that mice deficient in Mn-SOD2 develop normally *in utero*, but die soon after birth with dilated cardiomyopathy [6]. In contrast, mice deficient in cytosolic superoxide dismutase (Cu/Zn-SOD1) or extracellular SOD (Ec-SOD3) grow normally without a pathological cardiac phenotype [6, 69]. Importantly, these studies implicate the mitochondria as a significant source of ROS, which likely contribute to pathological cardiac remodeling. In parallel, they underscore the importance of antioxidant defense in attenuating pathological remodeling.

A number of clinical and experimental studies demonstrate increased generation of ROS in heart failure [70-73]. Moreover, animal models of heart failure document a decrease in the cellular antioxidant defense capacity [74, 75]. For example, Hill et al. [71, 72] provided evidence to support a progressive reduction in SOD, GPx, and catalase activity after experimental myocardial infarction. Moreover, plasma GSH levels have also been documented to decrease in patients

with acute myocardial infarction [76]. These observations indicate a common mechanism of oxidative stress induction after infarction and in heart failure, which likely reflect a combination of excessive ROS production and impaired antioxidant defense capacity. Importantly, the resultant oxidative stress appears to be a primary feature in the infarcted and non-infarcted myocardium during the early remodeling phase and persist in heart failure.

### *Oxidative Stress-Dependent Cardiac Remodeling*

Reactive oxygen species production and oxidative stress are primary features in both clinical and experimental myocardial infarction. Increasing evidence supports an important role for oxidative stress in cardiac remodeling [8, 9]. Specifically, ROS have been linked to apoptosis, interstitial fibrosis, and cardiomyocyte hypertrophy [40, 77, 78]; and the contribution of these processes to cardiac remodeling and secondary heart failure have been well-documented [8, 9]. Importantly, experimental reduction in ROS levels can attenuate cardiac remodeling following myocardial infarction [10, 62].

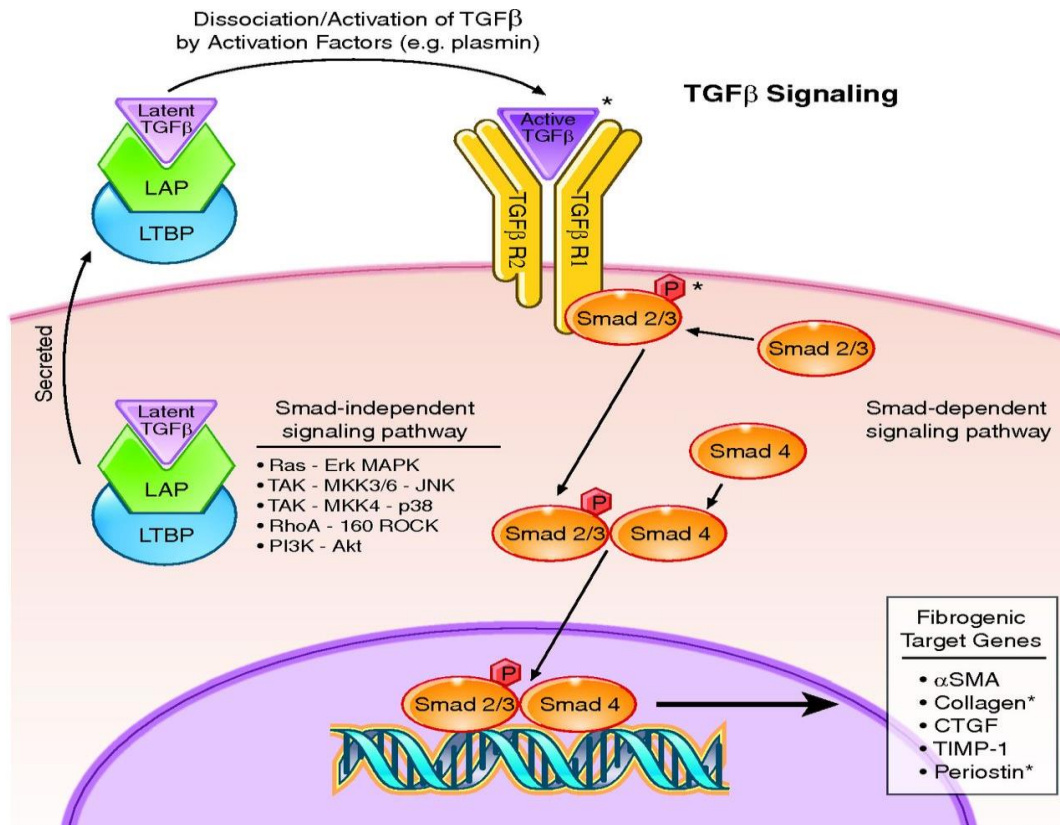
Oxidative stress driven by free radical formation in combination with reduced antioxidant defense capacity may be an important mechanism responsible for cardiac remodeling and progression to heart failure [71]. For example, Qin et al [9] documented cardinal signs of remodeling after experimental myocardial infarction, which were associated with increased NADPH oxidase activity, oxidative stress, and apoptosis. Importantly, treatment with apocynin, an NADPH oxidase inhibitor, reduced oxidative stress and apoptosis. In parallel, a study by Shiomi et al. [10] demonstrated that cardiac remodeling and heart failure following myocardial infarction could be attenuated with genetic overexpression of GPx. Their observations were associated with decreased apoptosis, interstitial fibrosis, and myocyte hypertrophy. In addition, genetic knockout

of Nox2, a catalytic subunit of NADPH oxidase abundantly found in cardiac tissue, reduced apoptosis, interstitial fibrosis, and myocyte hypertrophy after experimental myocardial infarction in mice [8]. Cardiac phenotypes from both studies display similarities providing further evidence that (1) oxidative stress drives pathological remodeling and (2) oxidative stress can occur from excessive ROS production and/or impaired antioxidant defense capacity.

Further, treatment with  $O_2^-$  to induce oxidative stress has been shown to promote apoptosis in cardiomyocytes *in vitro* [79]. Moreover, oxidative stress has been demonstrated to trigger apoptosis in several pathological conditions including: myocardial infarction, cardiomyopathies, and heart failure [80-82]. Importantly, death of viable cardiac myocytes is an important mechanism that contributes to the development of pathological remodeling [40]. Studies exploring the mechanisms of oxidative stress-induced apoptosis have shown increased expression of the proapoptotic factor *Bax* in the infarcted heart [83]. Further, activation of the intrinsic apoptotic pathway was shown to be associated with oxidative stress in an animal model of dilated cardiomyopathy [84]. Together, these findings suggest that oxidative stress can induce apoptosis in cardiomyocytes. Interestingly, TGF- $\beta$ -induced apoptosis is associated with oxidative stress and antioxidant treatment inhibited TGF- $\beta$ -dependent apoptosis [85-87]. These studies suggest that TGF- $\beta$  regulates apoptosis via mediation through oxidative stress. Importantly, both oxidative stress and apoptosis within the non-infarcted myocardium can be abolished with chronic treatment of antioxidants [88, 89].

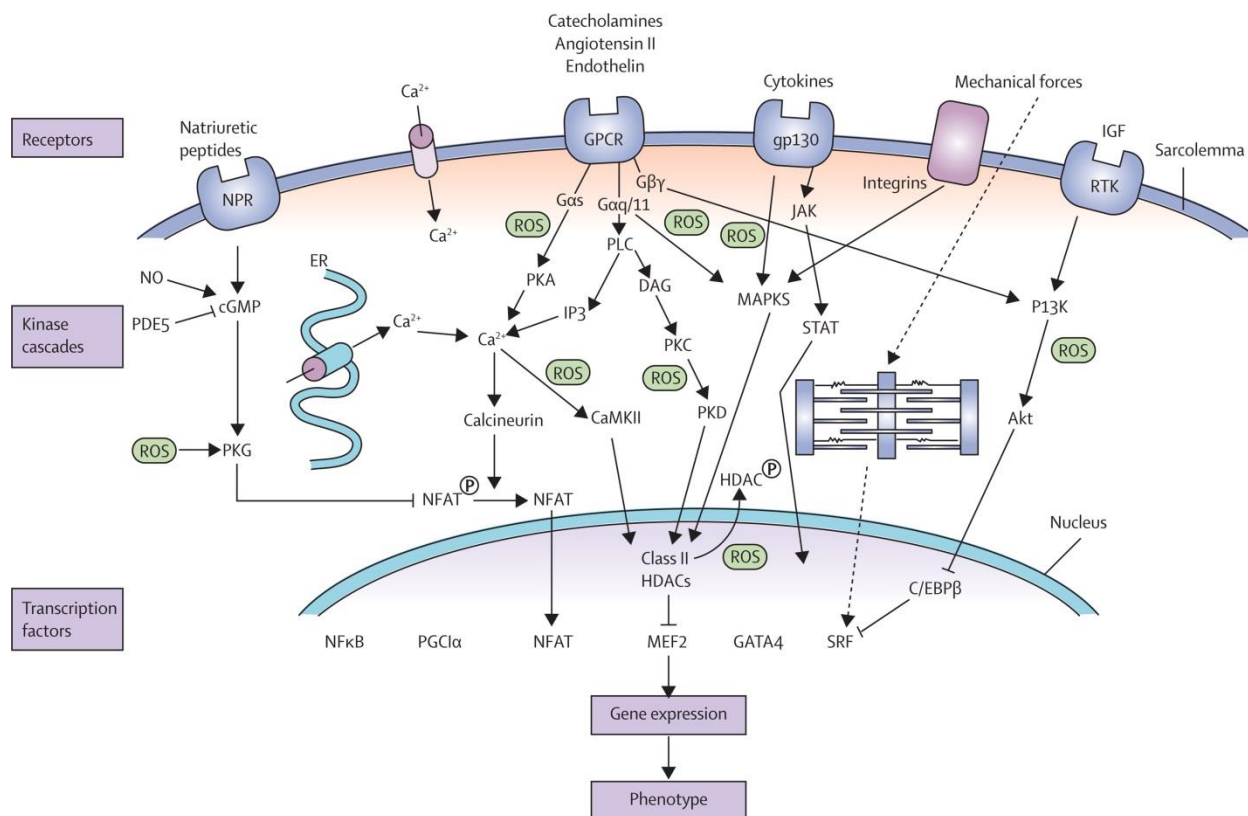
In the infarcted heart, fibrosis is a cardinal feature of cardiac remodeling, which is characterized as a scar in the infarct zone and interstitial fibrosis in the non-infarcted myocardium [57]. Importantly, oxidative stress has been further implicated in fibrogenesis not only in the my-

ocardium, but also in various tissues such as the lung and liver [90, 91]. Genetic or pharmaceutical inhibition of Nox4, another catalytic subunit of NADPH oxidase that is abundantly expressed in cardiac tissue, attenuated oxidative stress, and blocked TGF- $\beta$ 1 stimulated ROS production and subsequent activation of myofibroblasts [92]. These data indicate that fibrosis contributes to cardiac remodeling and that oxidative stress drives this process. Further, oxidative stress has been shown to directly regulate collagen synthesis and that Smad3 and CTGF are required for TGF- $\beta$ -dependent fibrosis [66]. Importantly, attenuation of oxidative stress with antioxidant treatment inhibited fibrosis [66]. Figure 4 depicts TGF- $\beta$ -dependent fibrosis mediated through Smads and CTGF. However, the precise mechanism responsible for oxidative stress-induced regulation of CTGF induction following a myocardial infarction remains to be identified.



**Figure 4.** Depiction of the CTGF gene as a downstream target of TGF- $\beta$  signaling in fibrogenesis [93]. Alpha-Smooth Muscle Actin ( $\alpha$ -SMA), Connective Tissue Growth Factor (CTGF), Latency Activated Protein (LAP), Latent TGF- $\beta$  Binding Protein (LTBP), Mothers Against Decapentaplegic Homolog (Smad), Tissue Inhibitor of Metalloproteinase (TIMP), Transforming Growth Factor-Beta (TGF- $\beta$ ).

In addition, ROS production has been associated with cardiac hypertrophy and secondary heart failure [10, 94]. For example, an *in vitro* study exploring the mechanisms driving pathological cardiac hypertrophy documented that TNF- $\alpha$  signaling induced hypertrophy, which was mediated through NF- $\kappa$ B in the presence of ROS [95]. At the transcriptional level, Mef2 and GATA4 have been implicated in pathological hypertrophy under conditions of pressure overload and oxidative stress (Figure 5). However, the underlying mechanisms of how oxidative stress regulates cardiac hypertrophy mediated through Mef2 and GATA4 activity following myocardial infarction are not currently known.



**Figure 5.** Depiction of the transcription factors Mef2 and GATA4 as downstream targets in the expression of pro-hypertrophic genes [96]. Abbreviations are listed on page XIX.

## Krüppel Like Factors

Krüppel-like factors (KLFs) are a subfamily of the large zinc-finger class of DNA binding transcriptional regulators. Most KLFs bind to consensus sequences such as the *CACCC* element or *GT* box in the promoter region of target genes. Further, protein-protein interactions regulate *trans*-activation and *trans*-repression of target genes in non-DNA binding regions of KLFs [97]. Moreover, KLFs are predominately expressed in the nucleus. As such, they are subject to post-translational modification and are responsible for recruitment of transcriptional co-activator/co-repressor complexes [98]. Seventeen mammalian KLFs have been identified so far and are represented in numerical order based on chronological identification [97]. In various tissues,

these factors have a wide range of important roles including: cardiac remodeling [99, 100], angiogenesis [101], monocyte activation [102], gluconeogenesis [103], and hematopoiesis [104].

### **KLFs in the Myocardium**

Several KLFs play pivotal roles in regulating many cardiac processes including: cardiac development, cardiac hypertrophy, cardiac metabolism, cardiac arrhythmogenesis [98], and cardiac fibrosis [105, 106]. For example, KLF13 expression is first detected at E9.5 in the atria and ventricles of developing embryos, which is reduced after birth. Deletion of KLF13 in *Xenopus* embryos results in septal wall defects. Further, deletion in murine embryos results in hypertrophic hearts [107]. In addition to KLF13, KLF3 has been implicated in embryonic cardiomyopathy and perinatal lethality. A missense mutation of the KLF3 gene results in embryonic lethality with hearts characterized by biventricular hypertrophy [108]. Surviving adult hearts were further characterized by dilated cardiac chambers. Together, these data provide a critical role for KLF13 and KLF3 in regulating normal cardiac development.

Hypertrophic stimuli such as angiotensin II induce a robust up-regulation of KLF5 expression. In contrast, targeted deletion of KLF5 blunts the angiotensin II-induced hypertrophic response [100, 109]. Since KLF5 expression is primarily restricted to cardiac fibroblasts, Takeda et al. [110] explored the mechanisms responsible for the interplay between cardiac fibroblasts and cardiomyocytes. They demonstrated that transverse aortic constriction induced KLF5 expression in both cardiac fibroblasts and cardiomyocytes, which was associated with cardiac hypertrophy and fibrosis. In contrast, KLF5 knockdown in cardiac fibroblasts, but not cardiomyo-

cytes, were less able to drive hypertrophic and fibrogenic signaling. In a subsequent series of experiments, they demonstrated that KLF5 expression in cardiac fibroblasts exerts an effect on cardiomyocytes mediated through paracrine action of insulin-like growth factor-1 (IGF-1).

In addition, pathologic hearts from KLF10 deficient male mice were characterized by septal wall hypertrophy, fibrosis, and myocyte disarray [111]. Interestingly, hearts from female mice deficient in KLF10 did not display signs of hypertrophy nor fibrosis, suggesting KLF10 exerts its effects downstream of the estrogen receptor. In parallel, mice with cardiac-specific deletion of KLF4 experienced high rates of mortality in response to pressure overload [112]. These hearts were characterized by cardiac hypertrophy, chamber dilation, fibrosis, and apoptosis. Taken together, these studies provide clear evidence in support of KLFs in regulating the hypertrophic and fibrogenic response in the myocardium.

### **General Functions of KLF15 in the Myocardium**

Recently, KLF15 has been implicated as an independent regulator of cardiac lipid metabolism. In support of this, KLF15 expression in the maturing mammalian heart tracks in parallel to the increases in lipid utilization [113]. Moreover, cardiomyopathies characterized by a decrease in lipid oxidation were linked to reduced KLF15 expression and this effect was reversed with unloading of mechanical stress on the myocardium [113]. Modeling substrate flux in the isolated heart, KLF15 deficiency resulted in a significant reduction in lipid oxidation which tracked in parallel with increased reliance on glucose oxidation [113]. Interestingly, the alteration in myocardial energy metabolism was associated with preserved contractile function without any change in hemodynamic indices. Further, altered substrate metabolism occurred without a



change in the expression of metabolic transcriptional regulators or co-activators such as peroxisome proliferator activated receptors (PPARs) and PPAR- $\gamma$  co-activator 1 (PGC-1), respectively. These data suggest that transcriptional regulators of cardiac metabolism may cooperate with KLFs to control energy metabolism. In support of this notion, a recently published study established the cooperative effects of PPAR- $\alpha$  and KLF15, wherein KLF15 binding to PPAR- $\alpha$  is required for PPAR- $\alpha$  mediated gene expression such as those involved in lipid metabolism [114].

Further, based on the expression patterns of KLF15, Jeyaraj et al. [115] speculated that KLF15 may regulate cardiac electrophysiology. They observed that KLF15 expression in the heart is rhythmic and that peak expression occurs during the transition from the inactive to active phase. To support this hypothesis, the investigators provided evidence for a molecular link to KLF15-dependent expression of a subunit required to maintain the transient outward potassium current ( $K_v$  channel-interacting protein 2; KChIP2). To further establish a regulatory role for KLF15 in cardiac electrophysiology, gain- and loss-of-function experiments provide additional evidence that KLF15 excess or deficiency result in perturbations of QT intervals, abnormal repolarization, and increased susceptibility to ventricular arrhythmias [115].

In addition to the established functions of KLF15 in cardiac lipid metabolism and electrophysiology, work by Fisch et al. [99] documented an expression pattern of KLF15 in the postnatal heart, which tracked in parallel to the time that classical hypertrophic gene markers (e.g., atrial natriuretic and B-type natriuretic peptides) are down-regulated. Moreover, KLF15 expression is down-regulated in response to hypertrophic stimuli such as angiotensin II, phenylephrine, and endothelin-1 [116] further establishing a regulatory role for KLF15 in cardiac hypertrophy. Cardiac-specific deletion of KLF15 is not embryonically lethal and mice do not display a patho-

logical cardiac phenotype [99]. However, these mice develop severe eccentric hypertrophy in response to pressure overload. Together, these results clearly implicate KLF15 as a negative regulator of pathological cardiac hypertrophy. To that end, continuing research investigating differential roles for KLF15 in cardiac remodeling are emerging.

### **KLF15 as a Negative Regulator of Cardiac Remodeling**

Overexpression of KLF15 potently inhibits three primary features of cardiac hypertrophy (e.g., hypertrophic gene expression, protein synthesis, and cell growth) in rat neonatal ventricular myocytes under both basal and stimulated conditions [100]. In contrast, KLF15 deficient mice do not display a hypertrophic phenotype at baseline, but these animals are exquisitely sensitive to stress and develop eccentric hypertrophy in response to pressure overload [100]. Importantly, transcriptional activity of two well characterized activators of hypertrophic remodeling (e.g., myocyte enhancer factor 2; Mef2, and GATA binding protein 4; GATA4) are under the strict control of KLF15 [99]. Importantly, KLF15 interferes with DNA binding of these transcriptional activators to the promoter region of their target genes, which is how they exert their pro-hypertrophic effects [97].

Mice overexpressing either Mef2A or Mef2C develop dilated cardiomyopathy, which is exacerbated under conditions of pressure overload [117]. In addition, mice overexpressing GATA4 result in severe cardiomyopathy and premature death, while cardiac myocytes in culture develop significant hypertrophy and protein accretion [118]. Interestingly, hearts from mice with cardiac-specific KLF15 knockout share a common phenotype with hearts from mice overexpressing Mef2 suggesting a negative relationship between the two factors.

Mef2 and GATA4 are known to interact and cooperate to serve as integrators for several upstream signaling pathways. Therefore, it is not surprising that enhanced activity of these pro-hypertrophic transcriptional activators in KLF15 deficient mice leads to marked dilated cardiomyopathy in response to stress. Further, during postnatal cardiac development, inhibition of Mef2 by KLF15 may serve as transcriptional “brake” for excessive Mef2 activity [99]. For example, Molkenin and Markham [119] demonstrated that Mef2 binding and activity increase during the postnatal period concomitant with an up-regulation of KLF15 expression [99, 120]. Moreover, gene products of Mef2 and GATA4 (e.g., atrial natriuretic and B-type natriuretic peptides) are markedly down-regulated at this time [120]. These data suggest that KLF15 serves to inhibit a pathological cardiac phenotype during maturation.

In addition to the known roles of KLF15 in regulating pathological cardiac hypertrophy, KLF15 deficient mice display interstitial fibrosis in response to pressure overload induced by aortic constriction when compared to wild type mice [106]. Moreover, there was a clear association between fibrosis and expression of connective tissue growth factor (CTGF) in isolated neonatal rat ventricular fibroblasts. Further, in response to TGF- $\beta$ 1 stimulation, KLF15 expression was markedly decreased with concomitant increased expression of CTGF. Together, this suggests TGF- $\beta$  stimulates the expression of CTGF and that KLF15 may negatively regulate the expression of CTGF.

In support of this hypothesis, reporter assays directly demonstrate a repressive effect of KLF15 on the CTGF promoter under both basal and TGF- $\beta$ 1 stimulated conditions [106]. Interestingly, KLF15 did not affect DNA binding of Smad3 to the CTGF promoter. Smad3 mediates signals from TGF- $\beta$  and has been implicated in fibrosis by up-regulating the expression of CTGF [121]. It is known that Smads and KLFs interact with the co-activator P/CAF and co-activators

such as P/CAF are often rate limiting. Therefore, Wang et al. [106] examined whether KLF15 competes with Smad3 to bind P/CAF and subsequently inhibit transcription of CTGF. Indeed, they demonstrated that KLF15 directly binds P/CAF competitively inhibiting Smad3 binding and thus preventing the association of P/CAF with Smad3 on the CTGF promoter.

Taken together, KLF15 appears to act concurrently in two different cardiac cell types to repress genes implicated in pathological cardiac remodeling. This is consistent with the notion that cardiac fibrosis is a prominent feature of pathological cardiac hypertrophy [105]. To that end, reports implicating KLF15 in regulating cardiac hypertrophy and fibrosis highlight an important role for KLF15 as a negative regulator of the pathological response to stress. This novel role for KLF15 makes it an attractive target for therapeutic interventions aimed at preventing cardiac remodeling observed following myocardial infarction.

### **Regulation of KLF15**

KLF15 has an intriguing expression pattern in the myocardium—it is not detected during embryonic development and its expression is very low during the early postnatal period. However, 30 days following birth, KLF15 expression is robustly up-regulated [120]. Under conditions of pathological stress such as pressure overload and valvular aortic stenosis in murine models and human subjects, respectively, KLF15 expression is dramatically reduced [99, 120]. Moreover, pharmacological agonists (e.g., phenylephrine and endothelin-1) known to induce a hypertrophic response in cardiac myocytes markedly reduce the expression of KLF15 [99]. Importantly, pathological conditions such as these are associated with significant oxidative stress. Therefore, it is reasonable to suspect that KLF15 may be sensitive to the cellular redox state.

In support of this, a recent report detailing expression profiles in cultured cardiomyocytes revealed a 50% reduction of KLF15 expression when exposed to H<sub>2</sub>O<sub>2</sub> [122]. In parallel, Vendov et al. [123] demonstrated that oxidative stress induced by NADPH oxidase in vascular smooth muscle cells was sufficient to drastically down-regulate of KLF15 expression. Taken together, these studies [122-124] indicate that KLF15 is sensitive to changes in cellular redox *in vitro* and that ROS-induced oxidative stress is a primary regulator of KLF15 expression in several cell types. However, the expression pattern of KLF15 and its sensitivity to the redox state *in vivo* following a myocardial infarction are not currently known.

### **Resveratrol Treatment**

Resveratrol (3, 5, 4-trihydroxystilbene) is a natural polyphenolic phytoalexin commonly found in the skin of red grapes and peanuts. A wide body of literature suggests a beneficial role for this plant extract in treating many chronic diseases such as cardiovascular disease [125], cancer [126], and diabetes [127]. Many cardiovascular diseases such as cardiomyopathies and heart failure secondary to myocardial infarction are associated with chronic elevations in ROS and/or impaired cellular antioxidant defense mechanisms [6]. Therefore, treatment with resveratrol may have beneficial effects in these redox-dependent models of disease due to its well-established antioxidant capabilities.

Moreover, several studies have demonstrated that resveratrol more effectively inhibits oxidative stress and damage when compared to conventional antioxidants [128, 129]. In parallel, resveratrol has also been shown to directly scavenge free radicals such as O<sub>2</sub><sup>-</sup> and OH<sup>-</sup> [130, 131]. Unfortunately, the ability of resveratrol to directly scavenge ROS is inferior to other well-

established antioxidants such as ascorbate and cysteine [132]. These studies suggest that the potent antioxidant effects of resveratrol must reside in an ability to up-regulate endogenous antioxidant defense mechanisms.

In support of this, data demonstrates resveratrol stimulates Nrf2 activation and induces robust expression of numerous antioxidant enzymes in cardiac tissue [133, 134] such as NADPH:quinone oxidoreductase-1 and -2, and  $\gamma$ -glutamylcysteine synthase (the rate-limiting enzyme for GSH synthesis), glutaredoxin-1 and -2, thioredoxin-1 and -2, and heme oxygenase-1 [135]. Taken together, these data clearly demonstrate a potent antioxidant role for resveratrol and that treatment with resveratrol may protect the myocardium from pathological remodeling driven by oxidative stress following a myocardial infarction.

Importantly, Chen et al. [83] reported that resveratrol protects cardiac myocytes from hypoxia-induced apoptosis. In parallel, Soner and Şahin [136] demonstrated that resveratrol provided a protective effect against H<sub>2</sub>O<sub>2</sub>-induced myocardial contractile dysfunction and aortic vasoconstriction. These data suggest that resveratrol provides a cardioprotective role, which is likely mediated by reduced oxidative stress. In agreement with previous reports, Lin et al. [137] provided additional support for a cardioprotective role of resveratrol. Their data showed that daily treatment with resveratrol reduced infarct size and improved both systolic and diastolic function. However, the mechanism of redox control of cardiac function and remodeling could not be ascertained from their data. For example, their study explored the effects of daily resveratrol treatment on the expression of a select few genes known to be implicated in the pathogenesis of myocardial remodeling. Specifically, they quantified mRNA expression of TGF- $\beta$ , ANP, and type I collagen. They reported a significant reduction in TGF- $\beta$  and ANP mRNA expression in resveratrol treated animals concomitant with no change in type I collagen mRNA transcripts. Based on

their data, they concluded that resveratrol exerted its beneficial effects by reducing TGF- $\beta$  and ANP mRNA levels. However, they did not explore any potential redox-dependent transcriptional regulation.

As such, the proposed study will explore the effects of daily resveratrol treatment on cardiac remodeling following myocardial infarction. Importantly, the redox-dependent transcriptional mechanisms responsible for cardiac remodeling driven by oxidative stress will be identified. Nrf2 protein expression and mRNA transcripts of antioxidant genes will be assessed as a surrogate marker of endogenous antioxidant capacity. Further, protein expression of KLF15 and mRNA transcripts of its known regulatory targets associated with cardiac remodeling will be assessed as an indicator of KLF15 signaling. Together, these data will provide support for the efficacy of antioxidant therapy in cardiac remodeling and attempt to elucidate the redox-dependent signaling pathways regulating cardiac remodeling.

### **Hypotheses and Specific Aims**

The normal structure and function of a healthy heart is vital to its mechanical and metabolic efficiency. Upon an ischemic insult that results in a myocardial infarction, a portion of the contractile myocardium used to generate ventricular pressure to eject blood is lost. As such, the myocardium undergoes a specific series of molecular and cellular changes to compensate for the loss of viable cardiac myocytes initiated by an intense inflammatory response. The early inflammatory response is responsible for clearing dead cells and matrix debris from the damaged site. Prompt resolution of the early degenerative inflammatory response is required to minimize the extent of the damage and promote the repair phase and restructure the myocardium.

Cardiac myocytes in the viable non-infarct region experience elevated mechanical stress and chronic oxidative stress. In response to increased mechanical and oxidative stress, myocardial cells express pro-hypertrophic and pro-fibrogenic gene programs. Exposure of cardiac myocytes and fibroblasts to chronic pathogenic stimuli such as mechanical stress and oxidative stress results in apoptosis of viable cardiac myocytes, excessive cardiac hypertrophy and ventricular dilation, and interstitial fibrosis, which may progress to secondary heart failure.

Recently, KLF15 has been identified as a negative regulator of the cardiac hypertrophic response to pathological stress. In parallel, KLF15 has been shown to attenuate the expression of the hypertrophic gene program by the inhibition of transcriptional activators GATA4 and Mef2. Further, KLF15 was shown to repress the transcription of the CTGF gene in response to pathological stimuli. Taken together, KLF15 acts as a transcriptional regulator to support cardiac health. However, cell culture experiments have demonstrated the KLF15 expression is dramatically reduced in response to oxidative stress. If the sensitivity of KLF15 to the redox state can be extrapolated *in vivo*, then presumably, its inhibition of hypertrophic and fibrogenic gene programs will be relieved to permit cardiac remodeling following myocardial infarction. To that end, alleviating oxidative stress may rescue the expression of KLF15 and attenuate the expression of these pathological gene programs.

**Therefore, in three integrated specific aims the purpose of this study will be to: (1) determine the effect of resveratrol on oxidative stress in the myocardium following a myocardial infarction, (2) determine the effect of resveratrol on the activity of KLF15 and its downstream targets, (3) determine the effect of resveratrol in preventing pathological cardiac remodeling following myocardial infarction (Figure 1). Together, these data will address the hypothesis that KLF15 plays a novel redox-sensitive role in the transcriptional**



**regulation of pathological cardiac remodeling associated with heart failure following myocardial infarction.**

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## CHAPTER TWO

### ANTIOXIDANT THERAPY ATTENUATES POST-INFARCT CARDIAC REMODELING BY DRIVING EXPRESSION OF KRÜPPEL-LIKE FACTOR 15

#### INTRODUCTION

Myocardial infarction (MI) results in severe biochemical, physiological, and cellular changes and often leads to alterations in the structure and function of the myocardium [1]. The structural changes, referred to as cardiac remodeling, are an adaptive response to the ischemic insult in order to maintain myocardial homeostasis during the acute phase of cardiac injury. However, pathological stimuli such as oxidative stress potentiate the magnitude of the remodeling response and are associated with progressive worsening of cardiac function [2, 3]. Hallmarks of cardiac remodeling following MI may include: loss of viable myocardium, interstitial collagen deposition, cardiac hypertrophy, cardiomyocyte apoptosis, and systolic dysfunction. Collectively, these remodeling events are identified as ischemic cardiomyopathy [3].

In an otherwise healthy heart, reactive oxygen species (ROS) are rapidly neutralized by a variety of endogenous enzymatic and non-enzymatic antioxidant systems [4]. In contrast, MI can result in oxidant production and suppressed oxidant clearance [5]. Further, increased oxidative stress due to accelerated oxidant production and attenuated scavenging systems has been linked to the pathophysiology of ischemic cardiomyopathy [6, 7]. For example, expression of the superoxide-generating enzyme, NADPH oxidase (Nox), increases after an MI, which is directly associated with cardiac remodeling and dysfunction [8]. In parallel, persistent oxidative stress in

hearts with ischemic cardiomyopathy is associated with depressed endogenous antioxidant defense and potentiated cardiac remodeling and dysfunction [7], suggesting that uncontrolled oxidative stress plays a key role in disease progression.

To combat oxidative stress, several mammalian genes harbor an antioxidant response element (ARE) in their promoter region such as the superoxide scavenging enzyme, superoxide dismutase (SOD) and the hydrogen peroxide scavenging enzyme, catalase (CAT) [9]. A major *trans*-activator of the AREs is the nuclear factor-erythroid 2-related factor (Nrf2) [9]. Many of the Nrf2-regulated antioxidant genes are essential in the prevention of cardiovascular disease [10] and their dysregulation is strongly associated with heart failure [11, 12].

A major end-product of Nrf2/ARE signaling is glutathione (GSH), the most abundant thiol antioxidant in cardiomyocytes. Importantly, GSH is a key determinant of redox signaling, detoxification of xenobiotics, modulates apoptosis, immune function, and fibrogenesis [13]. Interestingly, chronic oxidative stress plays a dominant role in GSH depletion in many redox-dependent diseases [14] and is associated with progressive worsening of the condition [15]. Further, several experimental studies have demonstrated that oxidative stress drives most, if not all, of the changes thought to contribute to cardiac remodeling [15-17]. Therefore, therapeutic interventions to restore the GSH pool and reduce persistent oxidative stress may attenuate the potentiated cardiac remodeling response and lessen the severity of cardiomyopathies.

Resveratrol (3, 5, 4-trihydroxystilbene) is a natural polyphenolic phytoalexin commonly found in the skin of grapes and peanuts. Importantly, resveratrol alleviates oxidative stress by directly scavenging ROS [18] and by driving Nrf2/ARE signaling [19]. Moreover, resveratrol use is becoming increasingly common for chronic diseases such as cancer [20], diabetes [21], and cardiovascular disease [22]. However, the redox-sensitive regulatory mechanisms involved in

pathophysiological gene expression associated with disease progression remain to be fully identified.

In response to oxidative stress, the transcriptional regulator Krüppel-like factor 15 (KLF15) is down-regulated in cultured cardiomyocytes [23] and vascular smooth muscle cells [24], which suggests it may extrapolate to *in vivo* conditions. Importantly, KLF15 has been identified as an antagonist to gene expression associated with cardiac remodeling in response to sustained pathological stimuli. More specifically, KLF15 inhibits the transcription of connective tissue growth factor (CTGF) by binding to its promoter [25], as well represses the transcriptional activity of myocyte enhancer factor 2 (Mef2) and GATA binding protein 4 (GATA), two important transcription factors associated with cardiac hypertrophy [26, 27]. However, the influence of persistent oxidative stress on KLF15-dependent signaling in a model of ischemic cardiomyopathy remains unknown. Accordingly, the purpose of this study was: (1) to determine the efficacy of resveratrol treatment on cardiac remodeling, (2) to determine the ability of resveratrol to drive Nrf2/ARE signaling, and (3) to determine the ability of resveratrol to manipulate KLF15 expression and signaling *in vivo* in a small animal model of ischemic cardiomyopathy. We hypothesize that chronic oxidative stress driven by the ischemic insult will down-regulate KLF15 signaling and permit the expression of cardiac genes associated with pathological remodeling.

## **METHODS**

### **Animals.**

Male Sprague – Dawley rats ~2 months old (Charles River Laboratories, Wilmington, MA) were housed in groups of 2 animals per cage, supplied with food and water *ad libitum*, and

maintained in a room at 20 – 22°C with a 12 hour photoperiod. All procedures were approved by the Georgia State University Institutional Animal Care and Use Committee.

### **Experimental Design.**

Myocardial infarction (MI) was surgically induced by permanent ligation of the left anterior descending artery (LAD) under isoflurane anesthesia. Animals that survived the surgery were randomly selected to receive daily intraperitoneal (i.p.) injections of resveratrol (ChromaDex, Irvine, CA; 10 mg/kg) dissolved in dimethyl sulfoxide (DMSO) or an equal volume of the vehicle, DMSO. Sham operated animals underwent the same pre- and post-surgical procedures as the MI group up to and including a left lateral thoracotomy and were also randomly selected to receive daily i.p. injections of resveratrol or vehicle. Treatments began after the animal recovered from the appropriate surgical procedure and continued for 28 days.

Animals were euthanized with carbon dioxide inhalation followed by a bilateral thoracotomy. Hearts were perfused with ice-cold phosphate buffered saline (PBS), weighed, and cut in the transverse plane at the level of the papillary muscles. The apical portion of the myocardium was cut at the mid-papillary muscle level and suspended in tissue freezing medium, rapidly frozen in 2-methylbutane cooled in liquid nitrogen and sectioned for histological and immunohistochemical analyses. The remaining left ventricular free wall consisting of both the viable myocardium and infarct scar was immediately frozen in liquid nitrogen and stored at -80°C for biochemical analyses.

## **Experimental Methods**

### *Left Anterior Descending Artery Ligation*

Animals were given a single pre-operative dose of Carprofen (5 mg/kg) subcutaneously (s.q.) one hour prior to surgery. Anesthesia was induced by exposing rats to 5% isoflurane gas and maintained at 2% and 1.5L O<sub>2</sub> per minute. Animals were then intubated using a 14G endotracheal tube (over the needle catheter), connected to a small animal ventilator (Harvard Apparatus, South Natick, MA), and secured to temperature controlled platform to maintain core body temperature between 35 and 37°C. An incision was made into the skin following the natural angle of the pectoralis major. The superficial and deep musculatures were reflected to expose the intercostal muscles. A transverse incision was made into the 4<sup>th</sup> intercostal space and the ribs were retracted exposing the thoracic cavity viscera. The LAD was permanently ligated using a 5-0 silk suture. Next, the thoracic cavity was closed using a 4-0 silk suture with a cross-stitch and the deep and superficial musculature was closed using a 5-0 silk suture with a simple continuous stitch. Finally, the skin incision was sutured using a 6-0 nylon suture with a simple interrupted stitch. Carprofen administration (s.q. 5 mg/kg) for post-operative pain alleviation was continued once daily for 3 days following the surgery.

### *Cardiac Morphology*

Transverse serial cryosections were cut at 10 μm at the mid-papillary level toward the apex using a cryostat (Leica CM1850, Leica, Germany) and adhered to superfrost microscope slides. Cryosections were then processed for hematoxylin & eosin (H&E) staining and Masson's trichrome staining using a commercially available kit (American MasterTech Scientific, Lodi, CA) according to the manufacturer's recommended protocol. Histological slides were visualized

using a VanGuard light microscope (VEE GEE Scientific, Kirkland, WA). Images were captured using ISCapture software (Xintu Photonics, Fujian, China). Cardiomyocyte cross-sectional area was calculated as the average area of approximately 50 myocytes from the border zone per H&E stained section from 3-5 sections per heart separated by approximately 400  $\mu\text{m}$ . Myocardial infarct scar size was calculated using the midline length method as previously described [28].

Briefly, the left ventricular midline was drawn at the center of the epicardial and endocardial surfaces along the length of the infarct scar that consisted of greater than 50% of the thickness of the myocardial wall. The infarct scar size was calculated by dividing the sum of the midline length measurements by the sum of the midline circumference from 5 Masson's trichrome stained sections per heart separated by approximately 400  $\mu\text{m}$  and multiplying by 100. Measurements of cardiomyocyte cross-sectional area and infarct scar size were calculated using ImageJ software (National Institutes of Health, Bethesda, MD).

#### *Terminal Deoxynucleotide Transferase dUTP Nick End-Labeling (TUNEL) Staining*

Transverse serial cryosections cut at 10  $\mu\text{m}$  were processed for apoptotic cell death detection using a commercially available TUNEL staining kit (PromoKine, Heidelberg, Germany) according to the manufacturer's recommended protocol. The apoptotic index was calculated as the number of TUNEL positive nuclei relative to the number of total nuclei from 10 fields per section and multiplied by 100.

#### *Immunoblotting*

Tissue samples from the left ventricular free wall were cut in the transverse plane. Ice-cold RIPA buffer (Thermo Scientific, Rockford, IL) supplemented with a protease inhibitor



cocktail (Thermo Scientific, Rockford, IL) was added to the tissues that were then homogenized using an electric homogenizer. The homogenate was then aliquoted and centrifuged at 10,000 g for 15 minutes at 4°C. Total protein concentration of the supernatant and whole tissue homogenate was measured using a bicinchoninic acid (BCA) assay (Thermo Scientific, Rockford, IL). Tissue homogenates were diluted in a 2X loading buffer containing glycerol and boiled at 100°C for 4 minutes. Thirty µg of protein was resolved on a 4 – 20% gradient sodium dodecyl sulfate (SDS) polyacrylamide gel at 120 V for 60 minutes. Proteins were then immobilized on a nitrocellulose membrane using a Trans-Blot Turbo transfer system (Bio-Rad, Hercules, CA) at 25 V for 30 minutes. Membranes were then blocked for 1 hour at room temperature in 5% non-fat dried milk (w/v) dissolved in tris-buffered saline with 0.05% Tween-20 (TBS-T) on an orbital shaker then stored overnight at 4°C. Following the block, membranes were probed with anti-Nrf2 (1:1000, R&D Systems) and anti-KLF15 (1:1000, Thermo Scientific, Rockford, IL) antibodies for 2 hours at room temperature on an orbital shaker. Following incubation with primary antibodies, membranes were washed with TBS-T (3 x 15 minutes) and then probed with the appropriate horseradish peroxidase conjugated secondary antibody (1:20000) for 1 hour at room temperature on an orbital shaker and washed. After the final wash, membranes were incubated in an enhanced chemiluminescent solution (Thermo Scientific, Rockford, IL) and immunoreactivity was visualized using a ChemiDoc imaging station (Bio-Rad Laboratories, Hercules, CA). Band density was quantified using Image Lab v4.0 software (Bio-Rad Laboratories, Hercules, CA) and normalized to Ponceau S stained bands.

### *Quantitative Real Time-Polymerase Chain Reaction*

Tissue samples from the left ventricular free wall were cut in the transverse plane. Trizol was added to the tissues (1 mL/100 mg tissue) that were then homogenized using an electric tissue homogenizer. Total RNA (1 µg) was reverse transcribed in a 20 µL final reaction volume using iScript Advanced cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's recommended protocol. Real time PCR products were analyzed using Applied Biosystems 7500 Fast Real-Time PCR system. cDNA (5 µL of a 1:10 dilution) was amplified in a 25 µL reaction containing 1 nM gene-specific primer pair and iQ Sybr Green Supermix (Bio-Rad Laboratories, Hercules, CA). Primer sequences were designed using Primer3 and are listed in Table 2. Samples were incubated at 95°C for 15 minutes followed by 40 cycles of denaturation, annealing, and extension at 95°C, 60°C, and 72°C, respectively, with fluorescence recorded at the end of each annealing step. All reactions were performed in duplicate and the starting quantities of the genes of interest were normalized to 18S rRNA (Ambion, Austin, TX). The  $2^{-\Delta\Delta C_t}$  method was used to analyze alterations in gene expression and values were expressed as fold change relative to control [29].

### **Statistical Analysis**

An independent *t*-test or factorial analysis of variance (ANOVA; group by treatment) was used for comparisons followed by Bonferroni post-hoc tests using SigmaPlot v11.0 software (Systat Software, San Jose, CA). Values are reported as mean  $\pm$  SEM. Statistical significance was accepted at  $p \leq 0.05$ .

## RESULTS

### Chronic Ischemia Induces Structural Remodeling of the Myocardium

Hearts from MI animals were significantly enlarged ( $p \leq 0.001$ ) (Table 2) and displayed a significant loss of viable myocardium to the left ventricular free wall (Figure 6A). This loss of contractile tissue was replaced with fibrous scar tissue containing a significant quantity of collagen (Figure 6A). Moreover, cardiomyocytes in the MI border zone increased in average cross-sectional area (CSA;  $p \leq 0.001$ ) (Figure 7B). Additionally, cardiomyocytes adjacent to the border zone had significantly more apoptotic nuclei in untreated infarcted hearts ( $p \leq 0.001$ ) (Figure 7B) that was associated with an induction of the apoptotic signaling pathway (Figure 9A-C). Importantly, resveratrol treatment markedly attenuated the infarct scar (by 18.2%,  $p = 0.027$ ; Figure 6B), the increase in average CSA of cardiomyocytes (by 13.9%,  $p = 0.004$ ; Figure 7B), and the number of apoptotic nuclei (by 24.3%,  $p \leq 0.001$ ; Figure 8B).

### Resveratrol Restores Nrf2 Protein Expression and Mediates Cellular Redox State

Next, we assessed Nrf2 expression to determine if the beneficial effects of resveratrol treatment were associated with the induction of antioxidant defense mechanisms. In hearts from untreated MI animals, Nrf2 expression was reduced by 51.4% ( $p = 0.039$ ) (Figure 10B). Interestingly, Nrf2 expression in hearts from resveratrol treated MI animals was not different from untreated sham operated animals ( $p = 0.383$ ) (Figure 10B).

As a surrogate marker of Nrf2/ARE activity, we quantified mRNA expression levels of key Nrf2/ARE-dependent oxidant scavenging enzymes. MI tended to increase the expression of the superoxide-generating NADPH (Nox)-4 subunit by 300% ( $p = 0.12$  [ns]) (Figure 11D). In contrast, this increase was attenuated in hearts from resveratrol treated MI animals. Interestingly,

these hearts exhibited a 400% increase in the mRNA transcripts of the cytoplasmic form of the superoxide scavenging enzyme (SOD)-1 (Cu/Zn-SOD1) ( $p = 0.08$ ) (Figure 11A). In contrast, these hearts displayed no significant change in mRNA transcripts of the mitochondrial form (SOD)-2 (Mn-SOD2) ( $p = 0.325$ ) (Figure 11B). Moreover, the mRNA expression of the hydrogen peroxide scavenging enzyme, CAT, is robustly increased in resveratrol treated infarcted hearts ( $p = 0.027$ ) (Figure 11C).

Hearts from resveratrol treated MI animals displayed an increase in the mRNA expression of the two glutathione synthesizing enzymes, glutamate-cysteine ligase catalytic subunit (GLCL;  $p = 0.11$  [ns]) (Figure 12A) and glutathione synthase (GSS;  $p \leq 0.05$ ) (Figure 12B). Additionally, resveratrol treatment tended to increase mRNA transcripts of glutathione reductase (GSR) ( $p = 0.08$  [ns]) (Figure 12C). In contrast, resveratrol treatment had no effect on glutathione peroxidase (GPx-1) ( $p = 0.245$ ) (Figure 12D) mRNA expression. Interestingly, there was a 6-fold increase in the glutathione-dependent detoxifying enzyme (glutathione *S*-transferase; GST;  $p \leq 0.05$ ) (Figure 12E).

### **Resveratrol Drives KLF15 Protein Expression to Regulate Cardiac Gene Expression**

KLF15 protein expression was assessed to determine if resveratrol treatment could affect upstream transcriptional regulation of hypertrophic and fibrogenic genes. In hearts from untreated MI animals, KLF15 expression was reduced by 25.5% compared to hearts from untreated sham operated animals ( $p = 0.039$ ) (Figure 13B). Interestingly, hearts from resveratrol treated MI animals displayed a marked increase in KLF15 expression that was significantly higher compared to untreated sham operated animals ( $p = 0.017$ ) (Figure 13B).

Next, genes associated with pathological cardiac remodeling were assessed to establish a role for KLF15 in a model of ischemic cardiomyopathy. mRNA transcripts of ANP and BNP, markers of cardiac hypertrophy, were increased 13- and 19-fold, respectively, in hearts from untreated MI animals ( $p = 0.041$  and  $p = 0.01$ , respectively) (Figure 14A & B). Likewise, mRNA transcripts of TGF- $\beta$ 1 and CTGF, markers of cardiac fibrosis, were increased 4- and 6-fold, respectively, in hearts from untreated MI animals ( $p = 0.037$  and  $p = 0.024$ , respectively) (Figure 14C & D). Importantly, the gene expressions of ANP, BNP, TGF- $\beta$ 1 and CTGF were markedly attenuated in hearts from resveratrol treated animals ( $p = \text{ns}$ , compared to both untreated MI and sham operated animals) (Figure 14A-D).

**Table 1.** Real-Time PCR Primer Pairs

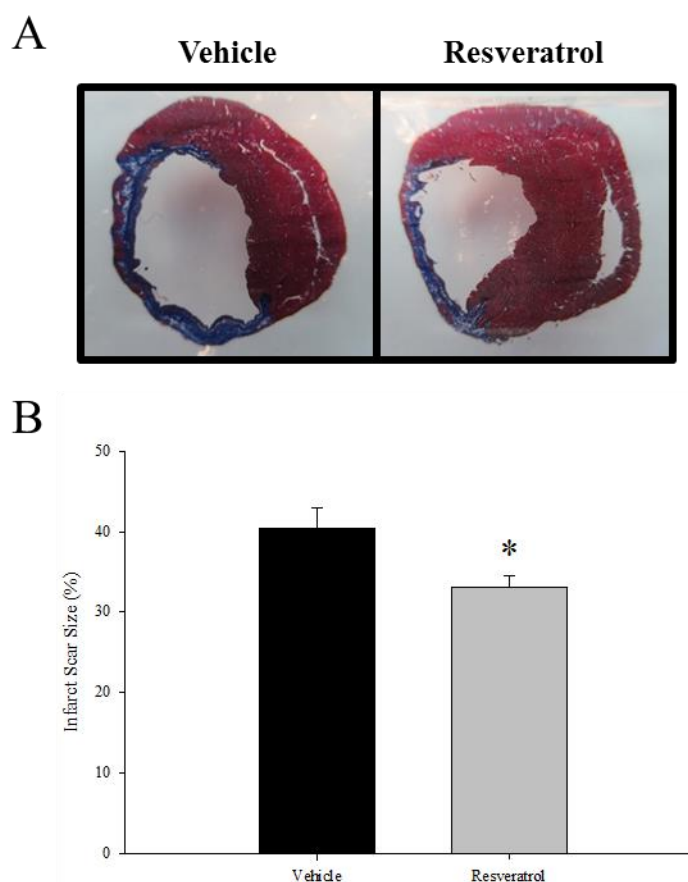
Gene	Accession No.	Primer Sequences (5' → 3')
ANP	NM_012612.2	F: TTCTCCATCACCAAGGGCTTC R: CTCTGAGACGGGTTGACTTCC
BNP	NM_031545.1	F: GCCAGTCTCCAGAACAATCAA R: AAGTCTCTCCTGGATCCGGAA
BCL-2	NM_016933.1	F: CTCTTCAGGGATGGGGTGAAC R: CAGCCTCCGTTATCCTGGATC
BAX	NM_017059.2	F: CGAATTGGCGATGAACTGGAC R: AGGACTCCAGCCACAAAGATG
CASP3	NM_012922.2	F: CTGGAATGTCAGCTCGCAATG R: TTCGGCTTTCCAGTCAGACTC
CAT	NM_012520	F: GGACCAGTACAACCTCCAGAAG R: ACTCCATCCAGCGATGATTACT
CTGF	NM_022266.2	F: GTCTCGCCGCCCTTCTTATTA R: TTCTGAGGGGAGAGAGACTGG
GCLC	NM_012815.2	F: TGGCCAGCCGTACGGAGGAA R: CAGGGCAGCCTAGCCTGGGA
GPx-1	NM_030826.3	F: ACACCGCTTACTTTCCCTCTG R: GCCATTCTCCTAGGGAAAGCA
GSR	NM_053906.1	F: GGGTGGTGTGCCACGGTTC R: ATAACGCTGCGGCTGGGCAA
GSS	NM_012962.1	F: TCACTGGACATGGGTGAAGA R: TCCATGAGGATGTAGGAGGC
GST	NM_177426	F: CACAAGATCACCCAGAGCAA R: CCATAGCCTGGTTCTCCAAA
Nox4	NM_053524	F: GGGCCTAGGATTGTGTTTGA R: CTGAGAAGTTCAGGGCGTTC
Cu/Zn-SOD1	NM_017050	F: CTTCTGTCGTCTCCTTGCTTTT R: CCTGTAATCTGTCCTGACACCA
Mn-SOD2	NM_017051	F: TGTGGCTGAGCTGTTGTAATCT R: GATGGCCTTATGATGACAGTGA
TGF- $\beta_1$	NM_021578	F: CTACTIONGCGCCAAAGAAGTCACC R: CTGTATTCCGTCTCCTTGTT

**Table 2.** Animal Body Weights (BW) and Heart Weights (HW)

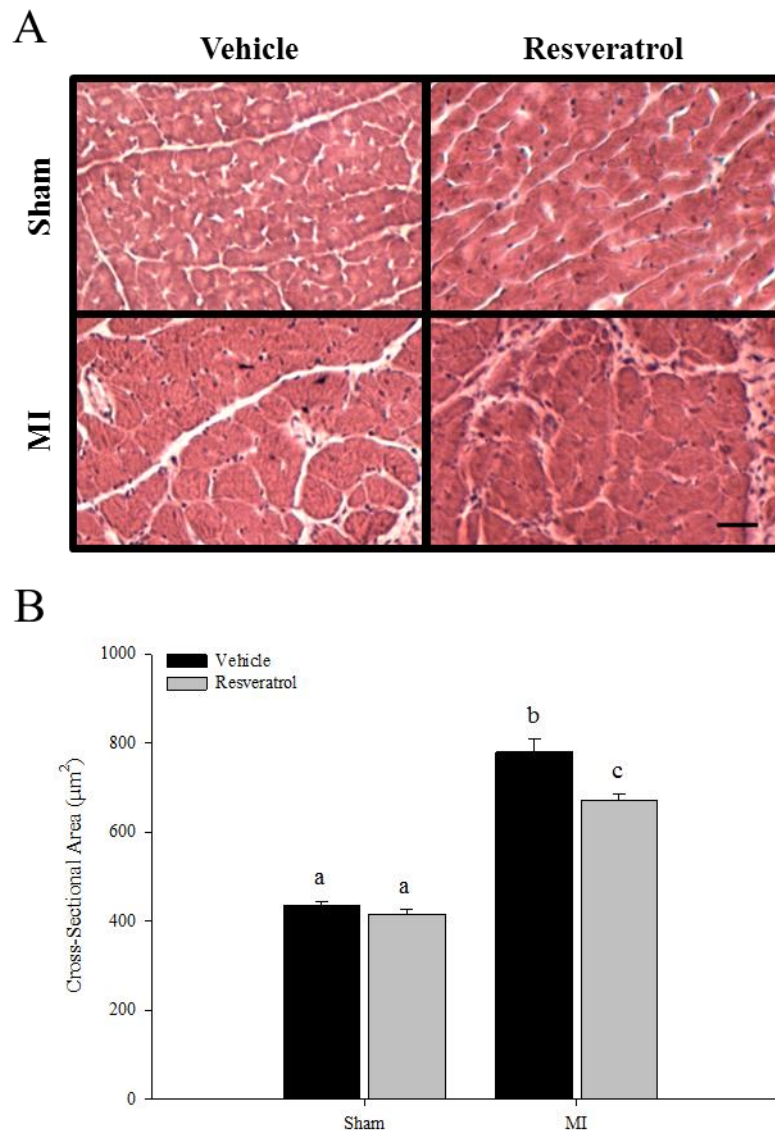
	Sham		MI	
	Vehicle	Resveratrol	Vehicle	Resveratrol
<i>N</i>	11	9	6	7
BW (g)	401.7±7.3	384.4±7.9	391.0±10.8	386.1±16.6
HW (mg)	1193.7±28.7	1206.9±27.7	1495.0±40.5*	1283.3±35.3
HW:BW	2.91±0.05	3.07±0.04	3.83±0.13*	3.34±0.07*#

Values are reported as means ± SEM. Significance was accepted at  $p \leq 0.05$ .

\*, compared to untreated sham operated animals. #, compared to untreated MI animals.

**Figure 6. Myocardial Infarct Scar Size**

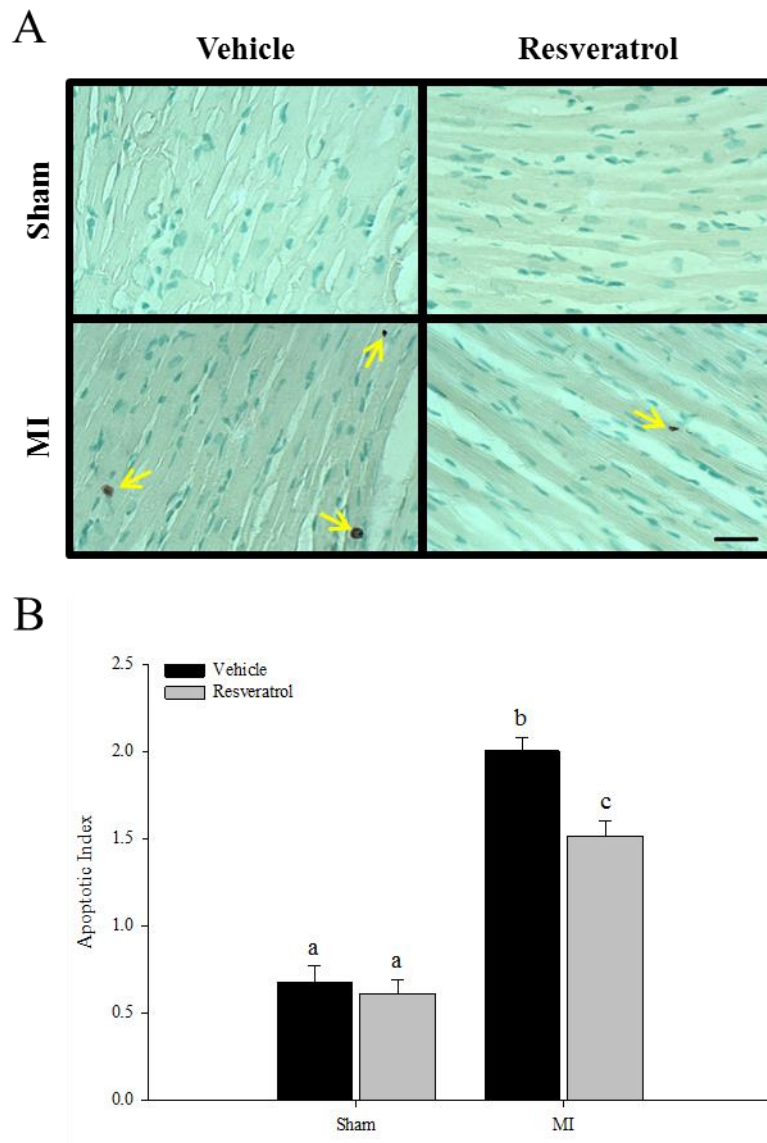
(A) Representative micrographs of infarct scar size in Masson's trichrome stained sections. (B) Bar graph representing the infarct scar as a percentage of the left ventricle. Values are expressed as means ± SEM ( $n = 5-6$  animals per group). Significance was accepted at  $p \leq 0.05$ . \*, compared to untreated MI animals.



**Figure 7. Cardiomyocyte Cross-Sectional Area**

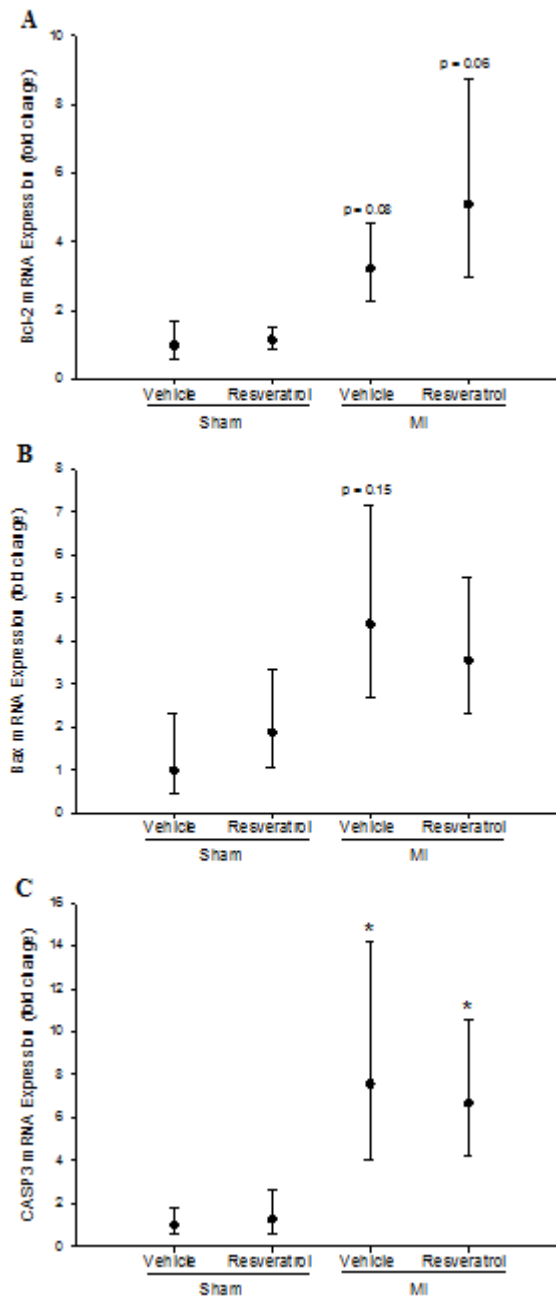
(A) Representative micrographs of cross-sectional area in hematoxylin & eosin stained sections. (B) Bar graph representing average cross-sectional area of cardiomyocytes. Values are expressed as means  $\pm$  SEM ( $n = 6$  animals per group). Significance was accepted at  $p \leq 0.05$ . Groups with the same letter are not statistically different. Images were magnified using a 10X objective and the magnification bar represents 100  $\mu\text{m}$ .





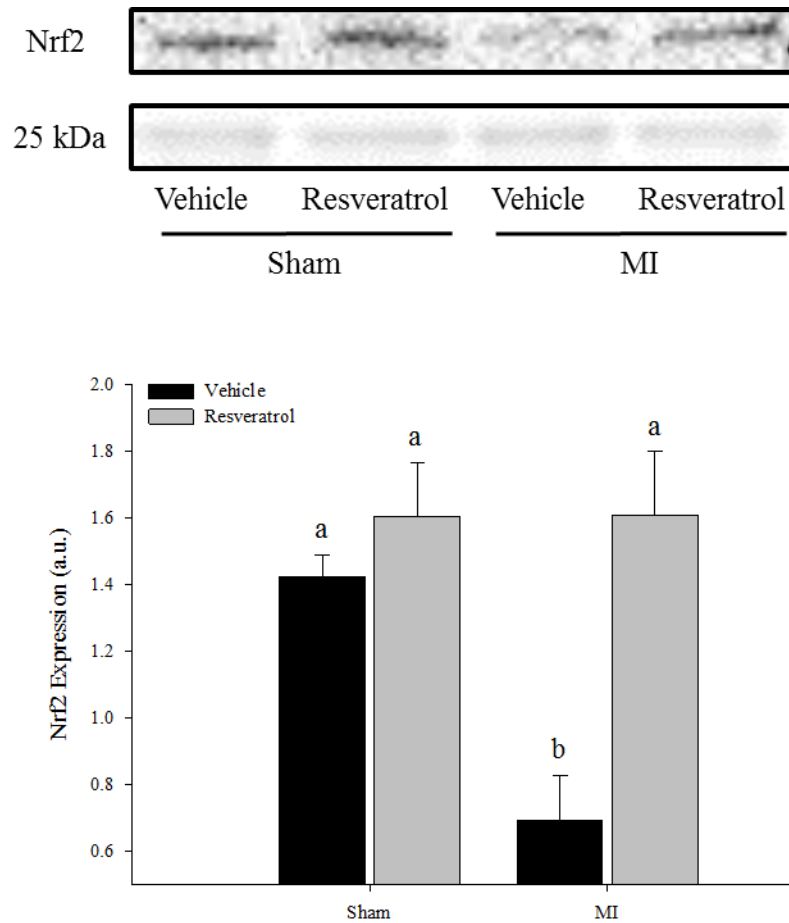
### Figure 8. Cardiomyocyte Apoptosis

Representative micrographs of cross-sectional area in TUNEL stained sections (A). Bar graph representing the apoptotic index (B). Values are expressed as means  $\pm$  SEM ( $n = 5-6$  animals per group). Significance was accepted at  $p \leq 0.05$ . Groups with the same letter are not statistically different. Yellow arrows indicate TUNEL positive nuclei. Images were magnified using a 40X objective and the magnification bar represents 25  $\mu\text{m}$ .



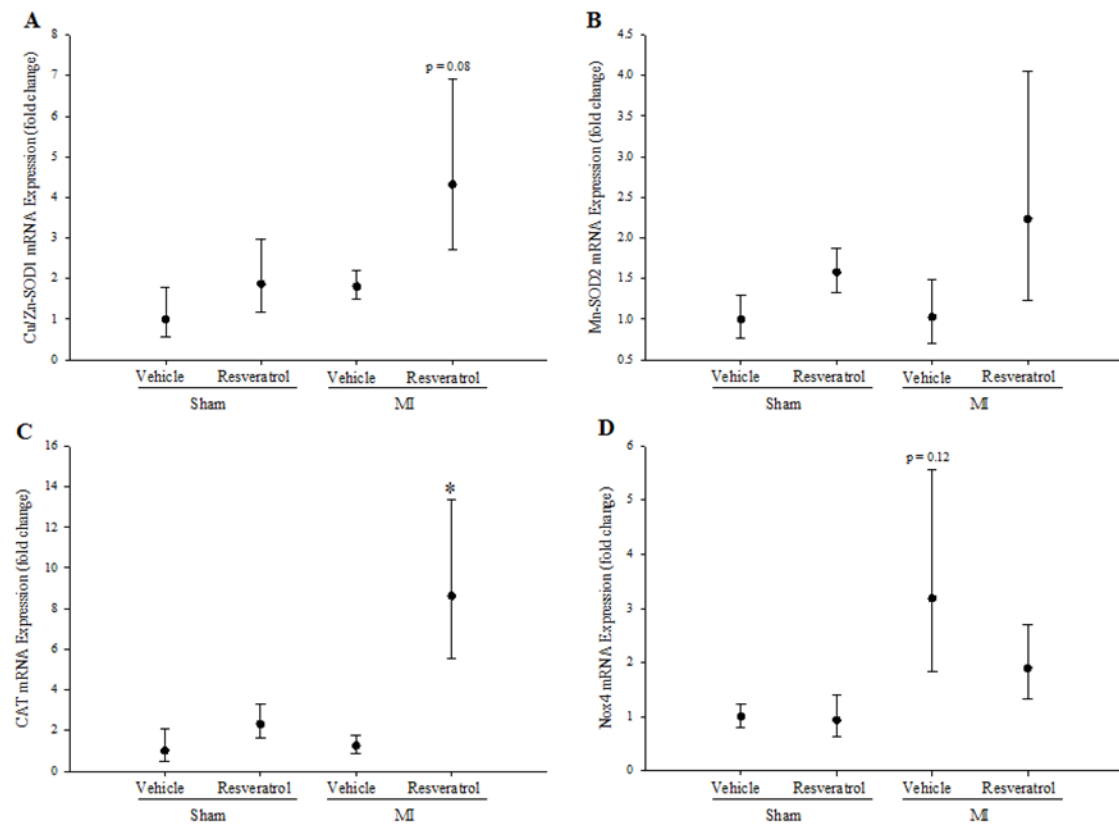
### Figure 9. Gene Expressions of Anti- and Pro-Apoptotic Factors

Real-time PCR analyses of the (A) anti-apoptotic factor, B-cell lymphoma 2 (Bcl-2), and pro-apoptotic factors, (B) Bcl-2-like protein 4 (Bax) and (C) Caspase-3 (CASP3). Data are represented as means  $\pm$  range of potential values based on the  $2^{-\Delta\Delta Ct}$  method and expressed as fold changes relative to untreated sham operated rats ( $n = 5-6$  animals per group). Significance was accepted at  $p \leq 0.05$ . \*, compared to untreated sham operated rats.



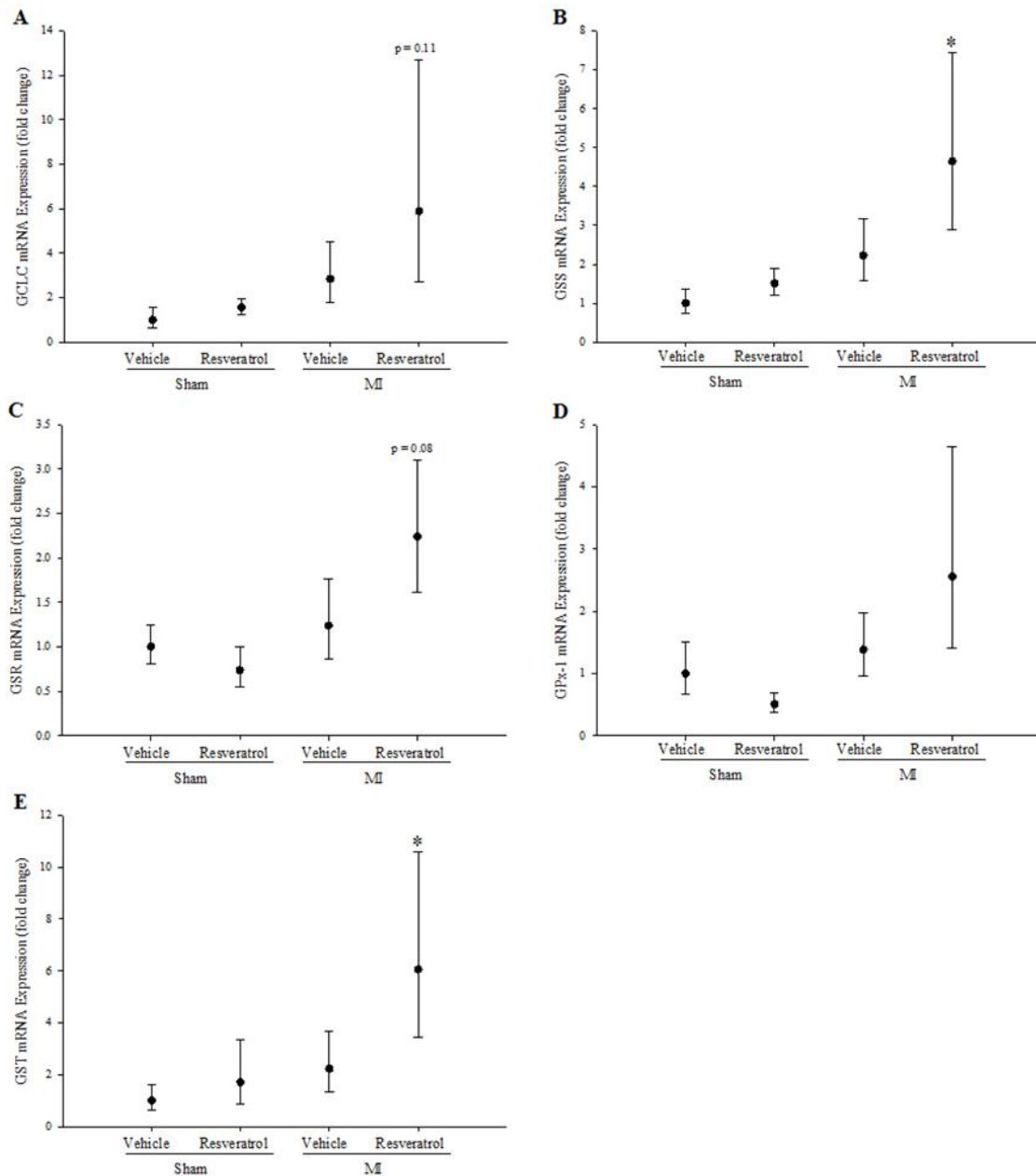
### Figure 10. Nrf2 Protein Expression

Representative immunoblot of Nrf2 protein expression and Ponceau S stained membranes. The bar graph represents normalized Nrf2 expression relative to the 25 kDa Ponceau S stained band density in arbitrary units. Values are expressed as means  $\pm$  SEM ( $n = 3-5$  animals per group). Groups with the same letter are not statistically different.



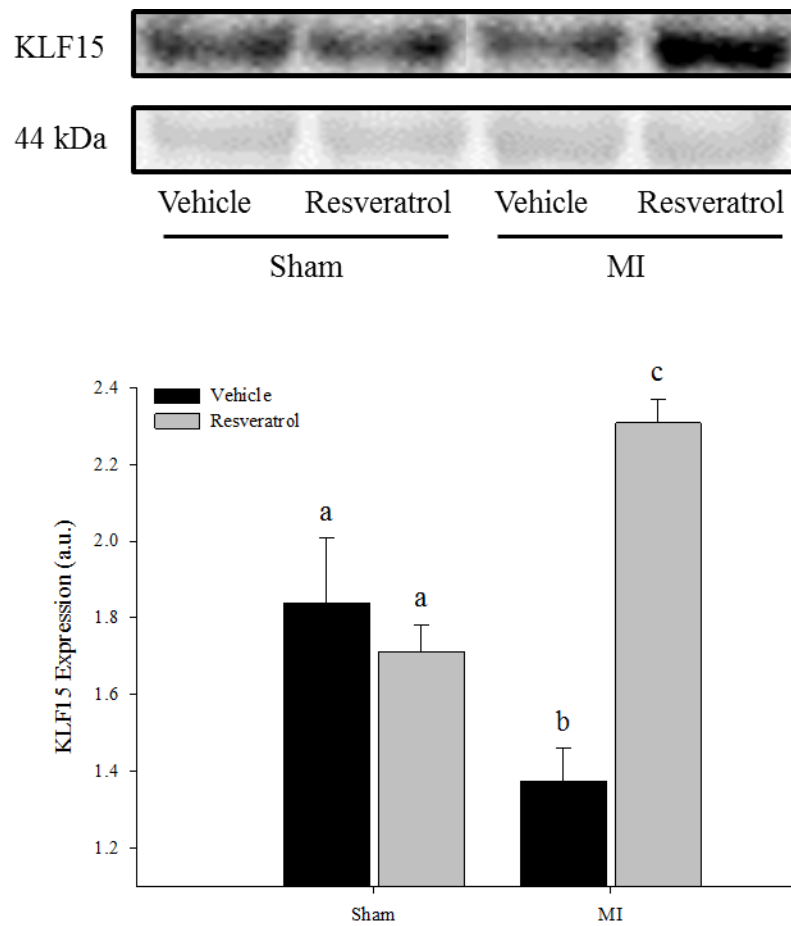
### Figure 11. Gene Expressions of Antioxidant and Oxidant Enzymes

Real-time PCR analyses of (A) Cu/Zn-superoxide dismutase-1 (Cu/Zn-SOD1) and (B) Mn-superoxide dismutase-2 (Mn-SOD2), (C) catalase, and (D) NADPH oxidase-4 (Nox4). Data are represented as means  $\pm$  range of potential values based on the  $2^{-\Delta\Delta C_t}$  method and expressed as fold changes relative to untreated sham operated rats ( $n = 4-6$  animals per group). Significance was accepted at  $p \leq 0.05$ . \*, compared to untreated sham operated rats.



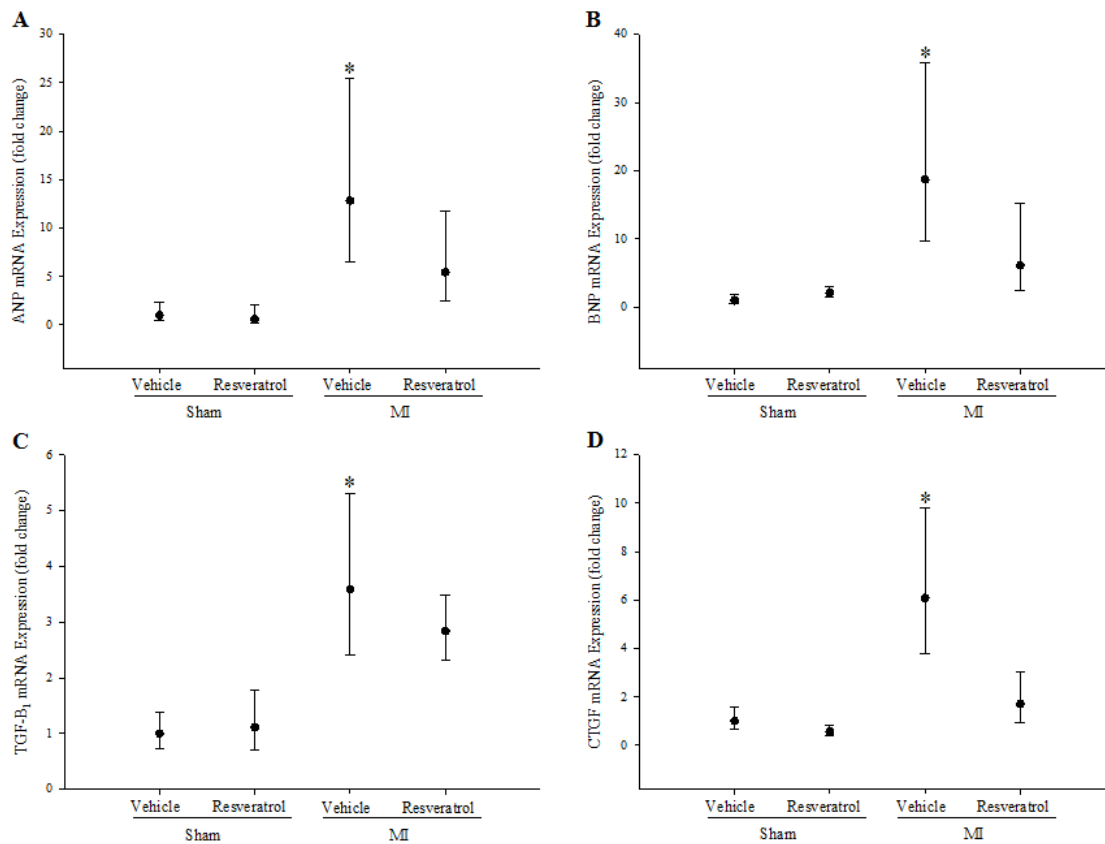
### Figure 12. Gene Expressions of Glutathione Synthesizing and Handling Enzymes

Real-time PCR analyses of (A) GCLC, (B) GSS, (C) GSR, (D) GPx-1, and (E) GST. Data are represented as means  $\pm$  range of potential values based on the  $2^{-\Delta\Delta C_t}$  method and expressed as fold changes relative to untreated sham operated rats ( $n = 4-6$  animals per group). Significance was accepted at  $p \leq 0.05$ . \*, compared to untreated sham operated rats.



### Figure 13. KLF15 Protein Expression

Representative immunoblot of KLF15 protein expression and Ponceau S stained membranes. Bar graph represents normalized KLF15 expression relative to the 44 kDa Ponceau S stained band density in arbitrary units. Values are expressed as means  $\pm$  SEM ( $n = 4-6$  animals per group). Groups with the same letter are not statistically different.



**Figure 14. Gene Expressions of Hypertrophic and Fibrogenic Markers**

Real-time PCR analyses of (A) atrial and (B) B-type natriuretic peptides, and (C) transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) and (D) connective tissue growth factor (CTGF). Data are represented as means  $\pm$  range of potential values based on the  $2^{-\Delta\Delta C_t}$  method and expressed as fold changes relative to untreated sham operated rats ( $n = 5-6$  animals per group). Significance was accepted at  $p \leq 0.05$ . \*, compared to untreated sham operated animals.

## DISCUSSION

In this study, we confirmed that four weeks of chronic myocardial ischemia resulted in profound structural changes to the myocardium that included left ventricular dilation, dense fibrotic tissue formation, cardiomyocyte hypertrophy, increased apoptotic cardiomyocytes, and heart enlargement. These derangements were associated with decreased Nrf2/ARE signaling and increased Nox4 expression that likely lead to persistent oxidative stress and reduced KLF15 signaling. Attenuated KLF15 signaling may have permitted expression of several cardiac genes associated with potentiated remodeling of the myocardium. Importantly, daily treatment with resveratrol seems to have reduced chronic oxidative stress and ameliorated these derangements to the myocardium at the molecular, cellular, and organ levels.

In response to a myocardial infarction, fibrogenic and hypertrophic signaling pathways are activated and initially serve as an adaptive response to maintain cardiac function [3]. However, chronic pathological stimuli such as oxidative stress lead to continued remodeling and progressive deterioration of cardiac function [2, 8, 30]. Oxidative stress is associated with increased TGF- $\beta$ 1 signaling, natriuretic peptides (e.g., ANP and BNP), and increased apoptosis signaling have been reported in numerous and experimental studies [31-34]. Consistent with these reports, we demonstrated that infarcted hearts from untreated animals displayed a robust up-regulation of these fibrogenic, hypertrophic, and apoptotic markers. Importantly, we report that daily treatment with resveratrol reduced cardiac gene expression in response to chronic ischemia, which attenuated the magnitude of cardiac remodeling. Together, our data provide evidence that persistent oxidative stress potentiates cardiac remodeling and worsens the severity of ischemic cardiomyopathy.



In support of this notion, Looi et al. [35] demonstrated that expressions of Nox2 and Nox4 are elevated following a myocardial infarction. Further, infarcted hearts from these mice displayed a robust expression of fibrogenic and hypertrophic markers, and increased apoptotic nuclei. Interestingly, mice lacking the Nox2 isoform exhibited attenuated oxidative stress and reduced expression of several cardiac genes and apoptotic nuclei [35]. In parallel, Nox2 null mice displayed a significant reduction in the pathological phenotype associated with ischemic cardiomyopathy [35]. Similarly, Qin et al. [36] reported that pharmacological inhibition of NADPH oxidase with apocynin was sufficient to reduce oxidative stress, cardiomyocyte apoptosis, and remodeling of the myocardium. Together, these studies highlight a pivotal role for NADPH oxidases in inducing oxidative stress following a myocardial infarction and that genetic and pharmacological inhibition of the catalytic subunits of NADPH oxidase is sufficient to alleviate oxidative stress and cardiac remodeling. Moreover, Shiomi et al. [12] demonstrated that mice genetically engineered to overexpress the glutathione-dependent detoxifying enzyme, glutathione peroxidase, were protected from cardiac remodeling and failure following a myocardial infarction. Taken together, these studies underscore the critical balance that oxidant generation and clearance play in establishing the redox state. Further, an imbalance between these systems and persistent oxidative stress appear to be responsible, at least in part, for driving cardiac remodeling through up-regulation of pathological cardiac gene expression.

Exposure to acute oxidative stress up-regulates the expression of Nrf2 and induces a robust Nrf2/ARE signaling cascade to protect the cell from oxidative damage [37]. For example, Nrf2 up-regulation protected cultured cardiomyocytes against oxidative stress and apoptosis [38, 39]. In contrast, these cardiomyocytes were highly susceptible to oxidative stress and apoptosis

following knockdown of the Nrf2 gene. Together, these studies provide direct evidence to support a critical role for Nrf2 in cardioprotection against oxidative stress and apoptosis. However, chronic exposure to oxidative stress reduces the expression of Nrf2 and depletes cellular stores of glutathione [14, 40, 41]. Here, we reported that chronic ischemia down-regulated the expression of Nrf2 and concomitantly increased the expression of Nox4. These data suggest that untreated infarcted hearts experience chronic accumulation of reactive oxygen species and oxidative stress, which explains, in part, the up-regulation of pathological cardiac gene expression. In contrast, treatment with resveratrol induced Nrf2/ARE signaling and attenuated the expression of Nox4. Taken together, these data suggest that resveratrol effectively increased the endogenous antioxidant systems and concomitantly reduced oxidant production.

To provide a further explanation for the efficacy of resveratrol treatment associated with oxidative stress alleviation, we demonstrated that the transcriptional regulator Krüppel-like factor 15 (KLF15) is redox-sensitive *in vivo*, which may regulate a host of pathological cardiac genes in a redox-dependent manner. For example, reports have documented that KLF15 regulates the transcription of CTGF [25], and the transcriptional activity of Mef2 and GATA4 [26, 27]. In support of this notion, we show that in conditions that likely induce persistent oxidative stress, KLF15 expression is markedly reduced, which may have permitted pathological cardiac gene expression observed in this study. Importantly, resveratrol treatment induced a robust expression of KLF15, which may, in part, explain the reduced cardiac gene expression and potentiated cardiac remodeling.

Interestingly, KLF15 may also negatively regulate persistent oxidative stress to promote its own expression. For example, a recent study by Yu et al. [42] reported that KLF15 regulates

TGF- $\beta$ 1 expression in conditions of mechanical and metabolic stress. Further, accumulating evidence demonstrates that TGF- $\beta$  signaling up-regulates Nox4 expression in a variety of cells [43-47]. The major mechanism involved in TGF- $\beta$ -induced Nox4 expression appears to be mediated through the Smad2/3 pathway. In support of this, Boudreau et al. [48] demonstrated that TGF- $\beta$ -dependent Nox4 expression and subsequent oxidant generation can be quenched by genetic knockdown of Smad3, suggesting Nox4 expression occurs downstream to the Smad pathway [49]. Additionally, TGF- $\beta$ 1 has also been reported to suppress the synthesis of glutathione by quenching the expression of GCLC, the rate limiting enzyme in GSH synthesis [50-52]. Taken together, it is likely that in conditions of persistent TGF- $\beta$  signaling, permitted by reduced KLF15 expression, may leave cardiomyocytes more susceptible to persistent oxidative stress. For example, oxidant production as a result of the initial ischemic insult drives TGF- $\beta$  signaling by reducing KLF15 activity, this, in turn, potentiates superoxide production while suppressing the capacity of cardiomyocytes to neutralize oxidants. In a perpetual cycle of TGF- $\beta$ -mediated oxidative stress, KLF15 activity remains repressed to permit the expression of hypertrophic and fibrogenic genes, which likely results in potentiated cardiac remodeling.

In support of this notion, we demonstrate that TGF- $\beta$ 1 and Nox4 mRNA transcripts are increased in conditions of reduced KLF15 expression. However, we show no appreciable decrease in GCLC mRNA transcripts. This may be due to an oxidative stress-mediated transcriptional activation of the GCLC promoter at a proximal AP-1 element [53]. Nevertheless, sustained TGF- $\beta$ 1/Nox4 signaling likely generates substantial oxidative stress to sufficiently deplete cellular GSH. In contrast, inducing the expression of KLF15 with resveratrol treatment attenuated both TGF- $\beta$ 1 and Nox4 expression and increased mRNA transcripts of GCLC. Taken together,

our data support a role for KLF15 in the regulation of oxidative stress in conditions of mechanical and metabolic stress mediated through a TGF- $\beta$ 1-dependent mechanism.

In conclusion, four weeks of chronic ischemia resulted in structural remodeling of the rat myocardium that was associated with reduced protein expressions of Nrf2 and KLF15. These factors are critical in providing cardioprotection and support a pivotal role in attenuating redox-dependent cardiac remodeling driven by persistent oxidative stress in response to a severe ischemic insult. Importantly, we demonstrate that daily treatment with resveratrol is sufficient to restore and up-regulate Nrf2 and KLF15 expression, respectively. As a consequence, potentiated cardiac remodeling and worsening of ischemic cardiomyopathy induced by persistent oxidative stress was ameliorated. Importantly, our data suggest that KLF15 may explain, in part, the efficacy of resveratrol treatment. While further experimentation is required, our data suggests that resveratrol treatment may provide a safe and therapeutically effective secondary treatment option for individuals who have had a myocardial infarction. Additionally, our data suggests that KLF15 may be an attractive therapeutic target to support cardiac health post myocardial infarction.

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## APPENDICES

### Appendix A: IACUC Original Protocol and Amendments

**Georgia State University  
Institutional Animal Care and Use Committee**

#### RESEARCH PROTOCOL FOR ANIMAL CARE AND USE

(Please **DO NOT** send initial submission as a PDF)

**For Office Use Only**

Date Received: January 20, 2015

Protocol Number: A15003

Protocol Title: Myocardial Infarction and Cardiac Regeneration

Principal Investigator: Dr. Jeffrey Otis

Veterinary Review Date: February 4, 2015

Revision Date: February 10, 2015

Final Approval Date:

Protocol Number Replacing:

Biosafety Approval Needed: Yes  No  Approval Date \_\_\_\_\_ Approval Number \_\_\_\_\_

Radiation Safety Approval Needed: Yes  No  Approval Date \_\_\_\_\_ Approval Number \_\_\_\_\_

Signature of Attending Veterinarian \_\_\_\_\_

Signature of IACUC Chair \_\_\_\_\_

The Institutional Animal Care and use Committee (IACUC) is by law responsible for ensuring that the use of animals at Georgia State University is performed according to the highest standards and in an ethical manner. This responsibility is shared with university faculty, staff, and students. The use of animals at the university is a privilege, not a right. Maintaining this privilege requires compliance with the following regulations, policies, and guidelines:

- [Animal Welfare Act Regulations](#)
- [Public Health Service Policy on Humane Care and Use of Laboratory Animals](#)
- [The Guide for the Care and Use of Laboratory Animals](#)
- [U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training](#)

The authority of the IACUC is derived from these laws and policies. The IACUC's role in institutional self-regulation ensures that animals are not subject to unnecessary pain and distress. Furthermore, by assuring compliance with these animal welfare laws and guidelines the IACUC also protects the investigator and the institution. The IACUC must review all aspects of the animal care and use program. The animal care and use program must include:

- A properly constituted and functioning IACUC
- Procedures for self-monitoring
- An adequate veterinary care program
- An occupational health and safety program
- A training program for personnel
- An environment, housing, and management program for animals
- Appropriately maintained facilities for housing and support

Central to the IACUC's mandated functions are (1) reviewing and approving animal use protocols submitted by investigators and (2) semi-annual program reviews and facility inspections.

This form is intended to facilitate review of requests to use animals for specific research, instruction, or biological testing projects.

- This completed form must be reviewed and approved by the IACUC before the project or course is initiated and before animals can be procured.
- After 3 years a new complete AUP must be submitted, approved and assigned a new number.
- The number of animals used must be declared annually and their documentation is the responsibility of the Principal Investigator.

The Georgia State University IACUC Policies and Procedures Manual can be found on line at: [GSU IACUC Policies and Procedures Manual](#) This document contains information on Completing the Protocol Form, Protocol Review and Approval, Required Training, The Occupational Health Program, Additional Considerations Pertaining to Protocols, and more.

**PLEASE COMPLETE A SEPARATE PROTOCOL FOR EACH DIFFERENT TYPE OF ANIMAL OR PROJECTS WITH DISTINCTLY DIFFERENT PURPOSES USING THE SAME TYPE OF ANIMAL (e.g. experimental protocol and breeding protocol).**

## SECTION 1. Basic Protocol Information

1.1 **PROTOCOL TITLE:** [Myocardial Infarction and Cardiac Regeneration](#)

1.2 **DATE:** [January 20, 2015](#)

1.3 **PRINCIPAL INVESTIGATOR:** [Jeffrey S. Otis, PhD](#)

1.4 **DIVISION:** [College of Education: Dept. of Kinesiology and Health](#)

1.5 **E-MAIL:** [jotis@gsu.edu](mailto:jotis@gsu.edu)

### 1.6 General Animal Information

1.6.1 **Over a period of three (3) years** I would like to use a total of 20 (number) animals.

1.6.2 Common name: [Rat](#)

1.6.3 Scientific name: [Rattus rattus](#)

1.6.4 I would like to begin using these animals on (date): [March 1, 2015](#)

1.6.5 I will obtain animals from (name of supplier): [Charles River](#). However, we will initially ask the greater GSU research community for unwanted animals (e.g., retired breeders, sentinels, etc.)

1.6.6 I will breed these animals:

Yes (If yes, justify in Section 14 "Animal Housing & Husbandry").  
Where will breeding stock be obtained? (Name of supplier):

No

### 1.7 List All Funding for the Proposed Animal Work:

<u>GRANT TITLE</u>	<u>GRANTING AGENCY</u>	<u>GRANT #</u>
Departmental Funds		

1.7.1. If Funding for the Proposed Animal Work is from the following sources, please submit the required documents listed below.

**NIH funding** - please attach the following:

Project/Performance Site Location(s)

- Project Summary Abstract
- Project Narrative
- Research Plan Attachments
- Specific Aims
- Research Strategy
- Vertebrate animals (grants.nih.gov/grants/olaw/VASfactsheet\_v12.pdf)

**NSF funding** - please attach the following:

- Cover sheet
- Project Summary
- Results from prior NSF support
- Explanation of Revisions
- Project Description
- Research Plan

### 1.8 **Three Year Renewal**

If this is a 3 year renewal, please answer the following questions. If not, please skip to section 1.10.

- 1.8.1 What is the number of the protocol this is replacing?
- 1.8.2 Do you presently have any live animals under this number?
- 1.8.3 If yes, will these animals be transferred to your new protocol once it is approved?
- 1.8.4 As this is a 3-year renewal, the IACUC requests that you provide a very brief description of the outcomes of the work conducted under the existing approval:

### 1.9 **Location of work on project**

- 1.9.1 Will any aspect of the study (course) or animal husbandry be conducted at **another institution**? If so, please name the institution: No
- 1.9.2 If yes, please provide the PHS Assurance number of the institution where this work will occur:
- 1.9.3 Has this proposal been approved by the IACUC of that institution?  
If yes, please attach a copy of the approved protocol and a copy of the dated and signed approval letter.

1.10 **Veterinary Care and Consultation:** Principal Investigators may discuss the proposed project with the Attending Veterinarian before submission of the application to the IACUC. Procedures involving more than momentary or slight pain or distress (USDA Pain Category "D" or "E") must be discussed with the Attending Veterinarian in the planning of the research project. All protocols and amendments will be sent to the Veterinarian for review of animal care issues after submission.

1.11 **Certification of Compliance:** I will comply with the procedures described in the NIH Guide for the Care and Use of Laboratory Animals, with PHS policy, the Animal Welfare Act, and the GSU IACUC Policies and Procedures Manual. I acknowledge responsibility for the procedures described and assure that the faculty, staff and students who perform these procedures are qualified (or will be adequately trained) to conduct them in a humane manner. Failure to comply may result in sanctions by the IACUC including, but not limited to suspension of research activities.

Signature of the Principal Investigator Jeffrey S. Otis

Date January 20, 2015

## SECTION 2. Lay Project Summary and Overview

### 2.1 Objective(s) (What are you doing?)

Please provide a brief statement, in LAY TERMINOLOGY understandable by someone with a college education, with no acronyms or scientific jargon, outlining the objective of the procedures of this protocol. Begin with a broad statement concerning the overall problem (e.g., “Pancreatic cancer kills ~50,000 Americans each year. We are addressing this problem by ...”). Include a statement of your experimental hypothesis or objectives. **Please do not submit your grant proposal abstract for this section.** Define all abbreviations the first time they are used and explain medical terms. You will be asked to provide a scientific summary of the project in a later section.

**\*\*\*, Please note that this new IACUC protocol is for a pilot study only. Here, we intend to fine-tune and demonstrate our proficiency with an established animal model of heart disease (ligation of the left anterior descending coronary artery). As such, we are only requesting 20 animals. Future amendments to this parent protocol will include requests for more animals and treatment interventions. That said, we have included our planned, future work in this section as it may assist the veterinary staff and IACUC reviewers.**

The American Heart Association has estimated approximately 450,000 individuals die each year in the United States from coronary artery disease and myocardial infarction (MI). Infarctions may cause cellular death to cardiac muscle cells, cause fibrosis, or lead to complete heart failure and death. By ligating the left anterior descending coronary artery to induce a MI, our ultimate goal will be to reduce the amount of tissue damage and improve cardiac performance by blocking molecular signaling pathways known to induce fibrosis in the heart (e.g., Wnt pathway). We will manipulate this pathway with the use of gene silence techniques or local administration of signaling peptides (again, to be described in amendments to follow).

### 2.2 Rationale and Significance (Why are you doing it?)

Please provide a brief statement about how contributions from your proposed work might be relevant to human/animal wellbeing or the expansion of knowledge. This must be written in LAY TERMINOLOGY, understandable by someone with a college education, with no acronyms or scientific jargon. **Please do not submit your grant proposal abstract for this section.** Define all abbreviations the first time they are used and explain medical terms.

According to the Centers for Disease Control and Prevention, 5.1 million people have been diagnosed with heart failure and about half die within 5 years of diagnosis. Medical costs associated with healthcare services and treatment for heart failure cost the nation over \$32 billion annually. Clearly, research focused towards improving cardiac function following heart failure is warranted and could potentially save lives and money.

### 2.3 Justification for the Use of Animals

Please justify why animals must be used instead of using methodology that does not require vertebrate animal use. Provide a brief statement justifying your use of animals in the proposed project.

Induction of MI and subsequent heart disease are a consequence of a multi-organ systemic response to the injury that includes metabolic, hematological, immunological, and pathophysiological involvement. Further, the heart is a multi-cellular organ which requires strict cellular communication in its native environment to execute the observed response to injury. Study of MI and cardiomyopathies requires the use of mammalian species as their physiology is similar to human physiology. Therefore, the effects of a MI and the development of heart disease can only be modeled *in vivo*.

## SECTION 3. Overall Animal Use Category Information

If the answer to any of the following questions is YES, please provide a brief explanation.

- 3.1 Will any technique be performed which will involve **prolonged physical restraint (> 30 minutes)** other than routine caging and handling? **Yes**  
If yes, describe the type, method and the length of time the animal is restrained: **While anesthetized, front and hindlimbs will be secured to the surgical platform with tape for approximately 45 minutes.**
- 3.2 Will any substance such as **Complete Freund's Adjuvant** or other adjuvants be injected which could cause chronic inflammation and/or pain? **No**  
If yes, describe what will be used, volumes, and the schedule for the injections:
- 3.3 Will it be necessary for live **animals to be removed** from the animal facility? **No**
- 3.4 Will this experiment involve **stress, pain, or abnormal behavior** in live animals, which cannot be alleviated with drugs because their use would interfere with the research goal? **No**
- 3.4.1 Will any adverse effects or overt **signs of illness** be expected? **Yes**
- 3.5 Will animals be subjected to **more than one major survival surgical procedure?** **No**  
*Major surgery penetrates and exposes a body cavity, produces substantial impairment of physical or physiologic function, or involves extensive tissue dissection or transection. Please note that routine injections are not considered surgical procedures.*
- 3.6 Will **food or fluid be restricted and/or regulated?** **No**
- 3.7 Will field investigations be employed? **No**  
If yes, please provide all the relevant permits:

## SECTION 4. USDA Animal Use Category Classification

**USDA Classifications and Examples of Pain Categories:**

**Classification B:** Animals being bred, conditioned, or held for use in teaching, testing, experiments, research, or surgery, but not yet used for such purposes.

**Examples:**

- Breeding colonies - Includes parents and offspring.
- Animals held under proper captive conditions or wild animals that are being observed.

**Classification C:** Animals upon which teaching, research, experiments, or tests will be conducted involving no pain, distress, or use of pain-relieving drugs.

**Examples:**

- Procedures performed correctly by trained personnel such as the administration of electrolytes/fluids, administration of oral medication, blood collection from a common peripheral vein per standard veterinary practice or catheterization of same, standard radiography, parenteral injections of non-irritating substances.
- Euthanasia performed in accordance with the recommendations of the most recent [AVMA Panel on Euthanasia](#), utilizing procedures that produce rapid unconsciousness and subsequent humane death.
- Manual restraint that is no longer than would be required for a simple exam; short period of chair restraint for an adapted nonhuman primate.

**Classification D:** Animals upon which experiments, teaching, research, surgery, or tests will be conducted involving accompanying pain or distress or leading to illness to the animals and for which appropriate anesthetic, analgesic, or tranquilizing drugs will be used.

**Examples:**

- Surgical procedures conducted by trained personnel in accordance with standard veterinary practice such as biopsies, gonadectomy, exposure of blood vessels, chronic catheter implantation, laparotomy or laparoscopy.
- Blood collection by more invasive routes such as intracardiac or periorbital collection from species without a true orbital sinus such as rats and guinea pigs.

- Administration of drugs, chemicals, toxins, or organisms that would be expected to produce pain or distress but which will be alleviated by analgesics.

**Classification E:** Animals upon which teaching, experiments, research, surgery, or tests will be conducted involving accompanying pain or distress or leading to illness to the animals and for which the use of appropriate anesthetic, analgesic, or tranquilizing drugs will adversely affect the procedures, results, or interpretation of the teaching, research, experiments, surgery, or tests.

**Examples:**

- Procedures producing pain or distress unrelieved by analgesics such as toxicity studies, microbial virulence testing, radiation sickness, and research on stress, shock, or pain.
- Surgical and postsurgical sequella from invasion of body cavities, orthopedic procedures, dentistry or other hard or soft tissue damage that produces unrelieved pain or distress.
- Negative conditioning via electric shocks that would cause pain in humans.
- Chairing of nonhuman primates not conditioned to the procedure for the time period used.

#### 4.1 Selection of Pain Category

Please classify the project according to the level of perceived pain / stress / distress experienced by the animal(s). Animals must be claimed under the highest class involved at any point prior to euthanasia or release. Highest Pain Category within this protocol: **D** (enter B, C, D, or E)

#### 4.2 Justification of Pain Category "E"

If category E is selected, please provide a scientific justification for withholding pain and/or distress relief.

#### 4.3 Monitoring of Animal Pain and Comfort Levels

4.3.1 Who will be responsible for monitoring the animals for pain and/or distress during the experimental procedure(s)?

[Russell G. Rogers, DAR staff, and GSU veterinarians](#)

4.3.2 How will the comfort level of the animals be determined?

[Pain or distress will be visually monitored and may include failure to groom, increased vocalization, hunched posture, decreased appetite and weight loss.](#)

[Surgery will be performed between 8AM and 10AM. Animals will then be monitored for any signs of discomfort and distress immediately after the surgery, 3PM and 6PM on the day of surgery. The next morning, animals will be monitored at 9AM, 12PM, 3PM, and 6PM; and, 9AM and 12PM the following morning.](#)

[The body condition scoring system for rats as published by Ullman-Cullere and Foltz \(Laboratory Animal Science, 49\(3\), 319-323, 1999\) and available online at \[http://www.aalas.org/pdfUtility.aspx?pdf=CM/49\\\_03\\\_15.PDF\]\(http://www.aalas.org/pdfUtility.aspx?pdf=CM/49\_03\_15.PDF\) will be utilized. Using this scale as a reference, we will implement a cutoff for euthanasia at a body condition of "1+." The body condition will be assessed at least twice a week and the body condition scores recorded in the DAR Laboratory Animal Care Record found in the animal room.](#)

4.3.3 In the event that an animal needs to be euthanized or removed from the experiment, please list the criteria for the decision. Describe the humane endpoint criteria to be applied and the frequency of monitoring for these humane endpoints:

[Should an animal require euthanasia because of significant stress, appropriate Georgia State University guidelines will be followed as identified in the GSU IACUC policies and procedures guideline located on the IACUC website \(<http://www.gsu.edu/research/iacuc.html>\).](#)

In addition, body weight will be recorded 3 times per week. Euthanasia will be recommended for any animal that loses 20% of their pre-injury body weight once normalized against a standard growth chart for young, growing animals. Animals in obvious distress (e.g., failure to groom, increased vocalization) that can not be alleviated with analgesia will be euthanized. In these animals, euthanasia will be performed via CO<sub>2</sub> inhalation and confirmed by thoracotomy.

Our conversations with Dr. Titterington suggest an expected mortality rate of 1 in 6 animals. These rates will be considered when calculating required animal numbers on future amendments. Dr. Titterington also notes that animals that survive 24 hours post-procedure generally survive through the duration of the 4-week recovery period.

4.3.4 Who will determine this action?

Russell G. Rogers, DAR staff, and GSU veterinarians

4.3.5 Will animal models with tumors be utilized? No

If yes, please review the following:

[http://ursa.research.gsu.edu/files/2013/04/GSU\\_IACUC\\_Policy\\_on\\_Mouse\\_Tumor\\_Burd\\_en.doc](http://ursa.research.gsu.edu/files/2013/04/GSU_IACUC_Policy_on_Mouse_Tumor_Burd_en.doc)

I will adhere to the standards specified in this policy.

Yes

No (If No, scientifically justify here):

## Section 5. Alternatives and Non-duplication

### 5.1 Explanation for the Consideration of Alternatives for Category “D” or “E” Animal Use.

Federal Regulations (The Public Health Service Policy and the [Animal Welfare Act](#)) and [University Policy](#) require assurance that this project does not unnecessarily duplicate research projects/courses performed at this or other institutions, and that the use of alternatives to live animal models and alternative procedures that may cause more than momentary or slight pain/distress (Class “D” and “E” procedures) to animals have been considered. The information in this section should include adequate information for the IACUC to assess that a reasonable and good faith effort was made to determine the availability of alternative models or methods.

**The following is a guide for answering 5.1.1 and beyond.**

**Your literature search is done as part of the OLAW requirement to address the “3Rs” (Refinement, Reduction, and Replacement) issues. For example:**

\* **Refinement** of procedures to eliminate or minimize pain or distress, the use of remote telemetry to decrease the distress of restraint; the use of humane endpoints.

Other examples of refinement are: ways to enhance the well-being of animals and the use of analgesics to decrease pain or anesthetics to decrease distress. These should be addressed only in Section 10.

For the literature searches:

**Ask "Am I using the least painful technique(s)?"**

- **Search terms:** Your species + animal/experimental model/technique + scientific keywords.

\* **Replacement** of live animals with non-animal procedures or a less sentient species. Examples include the use of non-animal models such as in-vitro work, cell culture, tissues culture, computer models or simulations.

**Ask "Do I need to use animals?"**

- **Search terms:** Your experimental model + simulation + in vitro +scientific keywords.

**Ask "Am I using the lowest species possible?"**

- **Search terms:** Your species + all applicable lower species (For example if using mice, specifically use "invertebrate", "fish" and "frog" in your search terms) + your animal/experimental model + scientific keywords.

**Note:** There are **NO** search terms that will direct the searcher to all examples of replacement or refinement. The onus is on the researcher to read and evaluate the literature.

**\*Reduction** in the number of animals used in the study. (**This only needs to be addressed in Section 9.2 of the protocol not here**). Examples include the use of shared control groups; preliminary screening in non-animal systems; innovative statistical packages.

**The selection of databases depends on the work performed and the species used in the protocol.** Samples of databases available through the Georgia State University Library [www.library.gsu.edu/database](http://www.library.gsu.edu/database) web pages.

· PubMed · Web of Knowledge · Biological Abstracts · Web of Science · PsychINFO · AltWeb  
NLM Gateway · AVAR · Galileo · AGRICOLA.

OVID (allows a search of multiple databases including Agricola, BIOSIS, CAB Abstracts, Medline, Zoological Record, etc.)

Please visit the Animal Welfare Information Center (**AWIC**) Literature Searching and Databases page ([http://awic.nal.usda.gov/nal\\_display/index.php?tax\\_level=1&info\\_center=3&tax\\_subject=184](http://awic.nal.usda.gov/nal_display/index.php?tax_level=1&info_center=3&tax_subject=184)) for a list of databases that can be used to search for alternatives. The AWIC site also recommends using the Literature Search Worksheet (<http://www.nal.usda.gov/awic/alternatives/searches/altwksht.pdf>) to assist in performing a successful alternatives database search. The worksheet helps to identify relevant searchable terms and concepts.

**5.1.1 Literature search for alternatives to painful procedures**

If you chose category "D" or "E" above, please do literature searches using the broadest database for your area of study, and provide a brief summary of the results obtained to verify that you investigated the use of alternatives to painful or distressful procedures. If you have not selected either category D or E, skip to section 5.2.

**5.1.1.1 Name of the databases used:** [Pubmed](#)

Date the databases search (es) were done: [January 14, 2015](#)

Did the search cover the entire date range of the databases with no restriction on dates?

Yes

No (If No, provide dates covered by the search(es) below)

Dates covered by the databases search(es):

**5.1.1.2 Search keyword(s) used:** include number of hits for combinations of terms. **You must use 'alternative' and 'animal welfare' in the search combinations for invasive or painful procedures** (See above for 3R criteria).

[myocardial infarction, anterior descending artery ligation, animal welfare, and alternative](#)

(1) [Myocardial infarction + animal welfare = 5](#)

(2) [Myocardial infarction + alternative = 3,061](#)

(3) [anterior descending artery ligation+ animal welfare = 0](#)

(4) [anterior descending artery ligation + alternative = 29](#)

**5.1.1.3 Summarize the information found in the hits below.** (Note: this should be a general summary. You do not need to go into the details of each hit but rather summarize hits by relevant groups).



The studies relevant to the research interest from the above searches describe *in vivo* mouse, rat, rabbit, and pig models. The *in vivo* studies track repair of the heart over time and the development of fibrotic tissue (i.e., remodeling).

- 5.1.2 Explain what alternatives exist in place of using animals for this protocol and provide a justification if these cannot be used.  
 Alternatives do not include less sentient species and are limited to *in vitro* systems. *In vitro* systems do not accurately reflect the *in vivo* condition as described in 2.3.

### 5.1.3 Other Sources of Information on Alternatives to Painful Procedures

- 5.1.3.1 Consultation with Experts: (Names, credentials, and dates): Dr. Jane Titterington, MD, PhD (Clinical and research fellow in the Division of Cardiology at Emory University under the advisement of W. Robert Taylor, MD, PhD.

Russell has met with Jane frequently during December 2014 and January 2015 for surgical training in the operation outlined in 8.1. In all, he has witnessed ~20+ successful surgeries (from induction of anesthesia through recovery). Training includes aseptic and surgical instrument preparation prior to the operation, pre- and post-op animal care, and surgical training. Importantly, while not named as a protocol associate, Dr. Titterington has agreed to serve as a consultant for this protocol and will provide guidance to Russell as needed on these procedures.

- 5.1.3.2 Scientific Meetings: Specify: No

*Note: A careful literature search is usually the best way to determine whether a proposed study is unnecessarily duplicative of previous work. However, it is ok to repeat a published experiment to make sure that it works in a different lab. This reason just needs to be stated as the justification.*

## 5.2 Consideration of non-duplication

- 5.2.1 Please provide a written assurance that the proposed work is not unnecessarily duplicative. This is a training protocol. Once proficient with the technique, all subsequent work using this model will be novel.

## Section 6. Method(s) of Euthanasia

- 6.1 Describe in detail the method of euthanasia (if any) you will use. If the method involves the use of pharmaceuticals, please specify agent, dose, and route of administration. Please note that methods of euthanasia must be in accordance with the most current [American Veterinary Medical Association Panel on Euthanasia](#). References must be cited to justify deviations from this document, or to justify use of cervical dislocation or decapitation without anesthesia. Should an animal require euthanasia because of significant stress or has completed their experimental time period, appropriate GSU guidelines will be followed. Euthanasia will be performed via CO<sub>2</sub> inhalation followed by thoracotomy to confirm death.
- 6.2 How will death be confirmed? thoracotomy
- 6.3 How will remains be disposed? Animal carcasses will be disposed by putting them in the DAR designated freezers within the animal facility.
- 6.4 If you chose Carbon Dioxide inhalation method above, please review the [GSU IACUC Carbon Dioxide Euthanasia Policy](#).  
 I will adhere to the standards specified in this policy.  
 Yes  
 No (If No, scientifically justify here):

## SECTION 7. Justification for Animal Species Selection

### 7.1 Animal characteristics

- 7.1.1 Species Name: [Rat](#)  
 7.1.2 Strain (if any): [Sprague-Dawley](#)  
 7.1.3 Age: [7 weeks to 24 months](#)  
 7.1.4 Sex: [Male](#)

### 7.2 This species has been selected because (check all that apply)

- Previous work in the biomedical literature validates the use of this species as an animal model for this disease or biological process.
- This is the lowest sentient species that provides appropriate size, tissue or anatomy of the proposed work.
- There is a large body of existing data that would need to be repeated if another species was used instead.
- Available reagents or research tools necessary for this research are unique to this species.
- Characteristics of this species make it uniquely suited for the proposed research. Explain:
- Other (Explain):

## SECTION 8. Animal Use Narrative

- 8.1 **Please describe in narrative form all experimental or instructional procedures to be performed on the animals.** Please note that it is not necessary to provide the details already provided elsewhere in the protocol (e.g. procedure descriptions, volumes of blood collected, dosages, routes of administration, use of aseptic procedures, etc.). **However**, it is important that one is able to ascertain what procedure or set of procedures is conducted on each group of animals. Include the time frames and intervals between procedures and describe the procedures in the order they will be performed.

[Animals will undergo ligation of the left anterior descending artery. This procedure will induce a myocardial infarction and has been described in greater detail in section 10. Animals will be sacrificed 4 weeks post surgery and hearts will be removed to assess damage, fibrosis, and several signaling components associated with heart disease.](#)

[Again, this initial protocol is for training purposes only. We will submit amendments that request more animals and treatments.](#)

## SECTION 9. Justification for the number of animals that will be used

- 9.1 Group sizes are expected to represent the minimum number of animals that are needed to achieve the scientific or instructional objectives. Please indicate all the methods used to determine these numbers.
- Statistical tools, such as power analysis, were employed to determine appropriate group sizes to ensure statistically valid outcomes. (Please retain print outs from calculations.)
- Previous experience with this experimental paradigm indicates this is the minimum number of animals needed.
- Consultation with a biostatistician
- This is a pilot study used to determine feasibility before proceeding with larger, more tightly controlled experiments.
- This is an instructional activity. This is the minimum number of animals needed based on class size and optimal student to instructor ratios.
- Other (Explain):

- 9.2 Using the specifics of your experimental plan demonstrate how the numbers of animals required to achieve your scientific objectives for this project were calculated. Include details of numbers of animals per group, control groups, treatment groups, pilot studies, and potential experimental failure. Information may be provided in the form of a table or flow chart. Justify the number of animals required for each procedure/experiment described in Section 10 using power analysis, if possible. For help with power analyses see: <http://www.psych.uni-duesseldorf.de/abtei-lungen/aap/gpower3/>

**Justify the number of animals requested:**

Animals outlined in this protocol will be strictly used as an instructional tool to provide more experience with the surgical technique prior to proceeding with a larger set of experiments. While animals in the experimental study will be purchased, we intend to ask the greater GSU research community to donate unwanted rats (e.g., retired breeders or sentinels, male or females).

## Section 10. Procedures

Please describe in detail all of the procedures you will be doing with animals below. Indicate whether infectious agents, chemical or physical restraint, radioactivity or adjuvants will be used. Describe possible/known side effects of each procedure.

**\*\*\*, Please note that we encourage the veterinary staff to watch the first several surgeries. Russell has witnessed 20+ surgeries at Emory and is confident he can perform them without incident. However, we feel it would be prudent to have vet staff present to confirm his proficiency or potentially provide improvements/refinements to his technique.**

### ***Induction of anesthesia***

Rats will be placed into an induction chamber and anesthetized via isoflurane inhalation (4% isoflurane and 500 mL O<sub>2</sub>/min) until the respiratory rate has fallen to a safe level and the animal fails to respond to toe pinch on all four paws.

### ***Intubation***

Rats will be anesthetized with an injection of a cocktail containing ketamine (40-50mg/kg) and xylazine (5-8mg/kg) IP. Once the rat has been intubated as described below, xylazine will be neutralized with yohimbine (2.1mg/kg) IP and the rat will be placed back on isoflurane. Per our discussions with the veterinary staff, once we become proficient with this intubation procedure, we will discontinue the use of this cocktail.

Rats will be placed on a plexiglass intubation stage with limbs taped down and teeth secured to the stage with fine line and a velcro strap. Using an endotracheal tube inducer (stylette), 14G endotracheal tube, and a small laryngoscope rats will be intubated as follows: The laryngoscope will allow access and provide light to visualize the vocal cords. When the vocal cords are open, the stylette and tube will pass through. Once passed the vocal cords, only the endotracheal tube will be further advanced and the stylette will be removed. A mirror will be used to ensure the animal was successfully intubated by observing fog accumulation as the animal breathes. Once intubated, the animal will be placed on an anesthetic ventilator (1.5 – 2.0% isoflurane and 500 mL O<sub>2</sub>/min). The ventilator will stimulate mechanical breathing via an intermittent positive pressure ventilation (IPPV) mode. Tidal volume will be set to 10 cmH<sub>2</sub>O with a mean airway pressure of 15 cmH<sub>2</sub>O at a frequency of 240 breaths per minute.

### ***Preparation of surgical site***

A heating pad underneath a sterile absorptive pad will be used to maintain core temperature at 35-37°C during the surgery. The site of the incision will be shaved (i.e., chest area) and sterilized via circular washes with 70% ethanol and betadine 3 times. Next, a sterile drape with hole removed exposing the surgical site will be placed over the animal ensuring a large sterile field. All surgical instruments, cotton swabs, and gauze will be sterilized via autoclave.

### ***Incisions and exposure of pericardial cavity***

A 1.5 inch incision will be made into the skin using a pair of surgical straight scissors starting at an area between the mid-axillary line and the superior aspect of the sternum (i.e., manubrium) to the inferior aspect of the sternum (i.e., xiphoid process). The skin will be separated from the underlying musculature to expose the pectoralis major using a pair of curved and blunted surgical scissors. Next, connective tissue (i.e., fascia) tethering the pectoralis major to the deep musculature will be removed such that the pectoralis major can be reflected forward using a pair of locking hemostatic forceps. The muscular immediately deep to the pectoralis major will be reflected backward using a pair of locking hemostatic forceps to expose the ribcage and the intercostal muscles. The apex of the heart will be identified by pulsating waves on the ribcage and the intercostal space closest to the apex of the heart will be selected as the site of entry into the thoracic cavity. **A small, transverse incision made to the left side of the 3<sup>rd</sup> and 4<sup>th</sup> intercostal spaces** will be made into the selected intercostal muscle followed by blunt force separation to ensure visceral organs are not damaged. The ribs will be retracted **with a small retractor** approximately 2 cm exposing the left lung and the heart. Next, the left lung will be retracted with a small (approximately 1.5 cm x 2.0 cm) piece of sterile gauze to expose the entire apex of the heart. The anterior portion of the pericardium will be removed prior to ligation of the left anterior descending coronary artery (LAD) to reduce the risk of developing pericarditis.

#### ***Ligation of left anterior descending (LAD) coronary artery***

A 5-0 silk suture attached to a C1 needle will be inserted into the myocardium just beneath the LAD at its superior branch. A surgical knot will permanently ligate the LAD. Induction of MI will be visually ensured via a color change of the anterior apical region of the left ventricle from a deep red to pink. In the event of excess bleeding, sterile cotton swabs will be used to remove the blood. If the cotton swabs are insufficient and bleeding continues, a cautery instrument will be used to stop the bleeding. **After successful ligation of the LAD, a 20G angio-catheter will be carefully inserted into the 6<sup>th</sup> intercostal space approximately 2 cm on the left side of the animal. Extreme caution will be taken to ensure no organs are damaged during the insertion of the chest catheter. After successfully inserting the chest catheter, the needle will be removed and the catheter tube will remain inserted into the thoracic cavity and proceed to closure of the thoracic cavity.**

#### ***Closure***

The gauze retracting the left lung will be removed and a check to ensure all organs are in their correct anatomical location and a chest catheter will be inserted (for use in the event of a pneumothorax) before closing the thoracic cavity. A 4-0 proline suture with a FS-2 needle will be inserted into the intercostal muscles in a single cross-stitch pattern to close the thoracic cavity. Next, the musculature deep to the pectoralis major will be sutured (with a 5-0 silk suture attached to a C1 needle) to the above pectoralis major at its most medial location just superior to the underlying musculature followed by suture of the pectoralis major at its most lateral location to the underlying deep musculature. Next, the skin will be sutured using a nonabsorbable 6-0 nylon suture attached to a C1 needle. **In addition, a topical anesthetic (1:1, 0.5% Bupivacaine: 2% Lidocaine) will be applied to the surgical site prior to closing the skin.**

#### ***Recovery from surgery***

After the skin has been completely sutured, isoflurane administration will cease and the animal will continue to be ventilated with oxygen until frequent spontaneous breaths are observed. The animal will then be removed from the ventilator (endotracheal tube still inserted) and placed into a nose cone to breathe oxygen. If the animal's respiratory rate is not sufficient, the animal will be placed back on the ventilator until adequate to be removed. Once the animal's respiratory rate is sufficient to be unhooked from the ventilator, the animal will then be extubated and remain in the nose cone to breathe oxygen. This will continue until the animal recovers from the anesthetic and is able to voluntarily move. In the event of a pneumothorax, a syringe will be connected to the chest catheter to remove any air trapped in the thoracic cavity. **After all air has been aspirated from the thoracic cavity and the animal has recovered from surgery, the animal will then be closely monitored for 2 hours for signs of distress and development of another pneumothorax. At this time, if no further air has developed in the thoracic cavity, the chest catheter will be removed.**

### **Analgesia**

On the day of the surgery, **carprofen (5 mg/kg, sc)** will be given post-procedurally to reduce pain. Analgesia treatment will continue once per day for the first 3 days after the surgery.

Animals will be sacrificed 4 weeks after surgery and their hearts will be removed for histological and biochemical assessment of the MI.

#### 10.1 **Prolonged physical restraint (> 30 minutes) that will be used under this protocol.**

10.1.1 How is the animal acclimated to the restraint device? **N/A**

10.1.2 Describe the monitoring of the animal during the time of restraint: **The animals will be restrained while under anesthesia. Animals will be visually monitored for changes to respiratory rate and/or core temperature.**

10.1.3 In the event of an animal welfare issue, what are the criteria for removal of the animal from the restraint device? **Animals will be euthanized (while under deep anesthesia) should complications from the surgery arise.**

#### 10.2 **Non-surgical procedures that will be used under this protocol.**

##### Non-surgical Procedures Description and Guidance

Describe each non-surgical procedure that will be used and state the maximum number of times each procedure will be done to any animal.

**N/A**

#### 10.3 **Surgical procedures that will be used under this protocol.**

##### Surgical Procedures Description and Guidance

Please indicate if surgical procedures will be used under this protocol

Yes

No (If No, then skip to Question 11)

10.3.1 Review the [ASEPTIC TECHNIQUE FOR ANIMAL SURGERY.](#)

I will adhere to the standards specified in this policy.

Yes

No (If no, scientifically justify here):

10.3.2 Review the [PREPARATION OF SURGICAL INSTRUMENTS, DEVICES, AND SUPPLIES POLICY.](#)

I will adhere to the standards specified in this policy.

Yes

No (If no, scientifically justify here):

10.3.3 Describe surgical procedures that will be used. Indicate type of surgery, provide a general description and indicate if it will be **survival (indicate major or minor) or non-survival** surgery.

10.3.3.1 Describe pre- and post-op procedures and monitoring: **Described above in section 10**

10.3.3.2 Will analgesics will be given pre- or post-procedurally? **Yes. Analgesia will be provided 1 hour before surgery, at surgery, and again 1 time per day for 3 days. In addition, a topical anesthetic (1:1, 0.5% Bupivacaine: 2% Lidocaine) will be applied to the surgical site prior to closing the skin).**

10.3.3.3 If analgesics are not used, justify why this is the case:

10.3.3.4 If sutures or wound clips will be used, indicate when they will be removed: **The duration of recovery from surgery is 28 days. Skin sutures, unless recommended by the vet staff, will not be removed from these animals in order to avoid additional stress.**

10.3.3.5 If multiple major surgeries in a single animal are proposed, please scientifically justify the necessity:

10.3.3.6. If multiple surgeries (major or minor) in a single animal are proposed, please indicate the minimum time interval between surgeries:

10.3.4 Describe any non-pharmacological control of pain post procedure such as:

- quiet darkened recovery area; and/or
- increased ambient warmth; and/or
- soft resting surface, etc.; or
- other; please explain:

## SECTION 11. Substance Administration

### 11.1 Substance Description

If anesthetics, analgesics, or any other substances are administered during the conduct of a procedure, please describe: ***Please copy and paste the set of questions as needed to list each additional substance(s) to be administered.***

11.1.1 Name of Substance: Isoflurane

11.1.2 Is this a non-pharmaceutical grade chemical or substance? No

If yes, please justify and indicate how sterility and purity are achieved:

11.1.3 Dose: Induction pre-intubation: 4%, Maintenance during procedure: 1.5 – 2% at 500mL O<sub>2</sub> per minute.

11.1.4 Route of Administration: inhalant

11.1.5 Frequency of administration: Once on the day of surgery

11.1.6 If applicable, state how anesthetic depth will be assessed: Respiratory rate and loss of toe pinch on all 4 limbs.

11.1.1 Name of Substance: Ketamine (to be used in combination with xylazine)

11.1.2 Is this a non-pharmaceutical grade chemical or substance? No

If yes, please justify and indicate how sterility and purity are achieved:

11.1.3 Dose: 40-50 mg/kg

11.1.4 Route of Administration: i.p.

11.1.5 Frequency of administration: Once on the day of surgery

11.1.6 If applicable, state how anesthetic depth will be assessed: Respiratory rate and loss of toe pinch on all 4 limbs.

11.1.1 Name of Substance: Xylazine (to be used in combination with ketamine)

11.1.2 Is this a non-pharmaceutical grade chemical or substance? No

If yes, please justify and indicate how sterility and purity are achieved:

11.1.3 Dose: 5-8 mg/kg

11.1.4 Route of Administration: i.p.

11.1.5 Frequency of administration: Once on the day of surgery

11.1.6 If applicable, state how anesthetic depth will be assessed: Respiratory rate and loss of toe pinch on all 4 limbs.

11.1.1 Name of Substance: Yohimbine

11.1.2 Is this a non-pharmaceutical grade chemical or substance? No

If yes, please justify and indicate how sterility and purity are achieved:

11.1.3 Dose: 2.1 mg/kg

11.1.4 Route of Administration: i.p.

11.1.5 Frequency of administration: Once on the day of surgery

11.1.6 If applicable, state how anesthetic depth will be assessed: NA

11.3.1 Name of Substance: Carprofen (analgesic).

11.3.2 Is this a non-pharmaceutical grade chemical or substance? No. Carprofen (Pfizer Animal Health)

If yes, please justify and indicate how sterility and purity are achieved:

11.3.3 Dose: Carprofen 5 mg/kg.

- 11.3.4 Route of Administration: subcutaneously
- 11.3.5 Frequency of administration: Carprofen will be administered at the time of anesthetic induction at 5 mg/kg subcutaneously with one additional dose (5 mg/kg) 24 hours after surgery.
- 11.3.6 If applicable, state how anesthetic depth will be assessed: N/A
- 11.1.1 Name of Substance: Bupivacaine (in 1:1 ratio with Lidocaine)
- 11.1.2 Is this a non-pharmaceutical grade chemical or substance? No  
If yes, please justify and indicate how sterility and purity are achieved:
- 11.1.3 Dose: 0.5%
- 11.1.4 Route of Administration: topical.
- 11.1.5 Frequency of administration: On the day of surgery
- 11.1.6 If applicable, state how anesthetic depth will be assessed: NA
- 11.1.1 Name of Substance: Lidocaine (in 1:1 ratio with Bupivacaine)
- 11.1.2 Is this a non-pharmaceutical grade chemical or substance? No  
If yes, please justify and indicate how sterility and purity are achieved:
- 11.1.3 Dose: 2.0%
- 11.1.4 Route of Administration: topical.
- 11.1.5 Frequency of administration: On the day of surgery
- 11.1.6 If applicable, state how anesthetic depth will be assessed: NA

## SECTION 12. Personnel and Their Experience and Training

List the experience and/or training of the personnel and the procedures that each will conduct below. If personnel are not experienced, please list the name of the individual(s) who will be responsible for training on all procedures. Please note that DAR and/or the IACUC reserve the right to observe procedures being performed prior to protocol approval.

If training has not been completed, visit <http://ursa.research.gsu.edu/ursa/compliance/iacuc/> and click on "Apply to Work with Animals". Under **Training** click on "Required Education Document" ([http://ursa.research.gsu.edu/repo/Required\\_Education.doc](http://ursa.research.gsu.edu/repo/Required_Education.doc)) to find out what requirements must be met in order to be considered approved personnel on this protocol. Click on "How to enroll in and use the AALAS Learning Library" link ([http://ursa.research.gsu.edu/files/2013/04/How\\_To\\_AALAS.doc](http://ursa.research.gsu.edu/files/2013/04/How_To_AALAS.doc)), if you are a first time user of the AALAS Learning Library. After the initial registration with the AALAS Learning Library, any future modules in the library can be accessed by following the "AALAS Learning Library training" link in this same section.

All employees of Georgia State University who work with vertebrate animals must enroll in the Medical Monitoring Program for Vertebrate Animal Exposure. Read [http://ursa.research.gsu.edu/files/2013/04/MMPVAE\\_Program\\_Description.pdf](http://ursa.research.gsu.edu/files/2013/04/MMPVAE_Program_Description.pdf) and carefully follow the instructions. To enroll click on the following link: <http://mmpvae.gsuapps.com/>

### **Principal Investigator:**

Name: Jeffrey S. Otis

Phone number and location: 404-413-8378, Office: G13 Sports Arena; Lab: G19 Sports Arena  
Procedures will do on animals: None

Experience: Dr. Otis has over 15 years of experience working with mouse and rat models – including survival surgery models.

GSU training completed: all as needed

Is this person enrolled in the Medical Monitoring Program for Vertebrate Animal Exposure?

yes

no .

**Graduate Students:**Name: [Russell G. Rogers](#)Phone number and location: [Cell: 770.365.5392, G01 \(office\) and G19 \(lab\) Sports Arena](#)Procedures will do on animals: [Russell will be responsible for all surgical procedures and post-procedural monitoring of the animals.](#)Experience (Indicate who will be responsible for training): [Russell has 5 years of experience working with rodent models, including small animal handling, surgical techniques, and post-procedural care. He has watched Dr. Jane Titterington, MD, PhD perform 20+ ligation surgeries and feels competent to perform the surgeries by himself.](#)GSU training completed: [All as needed](#)

Is this person enrolled in the Medical Monitoring Program for Vertebrate Animal Exposure?

 yes no**SECTION 13. Hazard Use**

All Hazardous Materials used must be identified.

For more information on the Biosafety program please click: [GSU Biosafety Program.](#)For more information on the Radiation Safety program please click: [GSU Radiation Safety.](#)For more information on Lab Safety and Hazard Communication please click: [GSU Lab Safety and Hazard Communication.](#)

13.1 Are you working with infectious agents and/or biologically-derived toxins?

 No Yes. I have approval from the Biosafety Committee

Date: \_\_\_\_\_ Approval #: \_\_\_\_\_

13.1.1 Describe all special precautions recommended for personnel who handle animals, equipment, or do housekeeping. Describe how contaminated caging or tanks, bedding, and equipment should be handled.

13.2 Does the project use any recombinant DNA (i.e. cloning/expression systems, viral vectors, etc.) material in animals?

 No Yes. I have approval from the Biosafety Committee

Date: \_\_\_\_\_ Approval #: \_\_\_\_\_

13.2.1 Describe all special precautions recommended for personnel who handle animals, equipment, or do housekeeping. Describe how contaminated bedding, cages or tanks or equipment should be handled.

13.3 Will any carcinogenic or toxic compounds to be used in animals?

 No Yes

13.3.1 Describe all special precautions and training recommended for personnel who handle animals, equipment, or do housekeeping. Describe how contaminated caging or tanks, bedding, and equipment should be handled.

13.4 Will any radioactive compounds be administered to the animals?

 No Yes. I have approval from the Radiation Safety Committee

Date: \_\_\_\_\_ Approval #: \_\_\_\_\_



13.4.1 Describe all special precautions recommended for personnel who handle animals, equipment, or do housekeeping. Describe how contaminated caging, bedding, and equipment should be handled.

13.5 Will Controlled Substances be used?

- No  
 Yes

13.5.1 Do you have a Controlled Substance Permit?

- No  
 Yes

## SECTION 14. Animal Housing and Husbandry

14.1 Will you breed these animals?

- No  
 Yes Please address the following: describe method of mating (harem, monogamous, et cetera); describe age of weaning; describe genetic monitoring and methods to assure inbred / outbred status; justify breeding versus obtaining commercially. If only one sex of offspring will be used in experiments, please justify. **A separate breeding protocol (Use breeding protocol form) may be submitted to generate animals for this and other experimental protocols.**

14.1.1 If breeding Genetically Modified Animals (GMAs), with their inherent potential for unanticipated phenotypes, please describe monitoring procedures for unexpected outcomes:

14.1.2 Please state if there is a plan in place for preservation of critical or irreplaceable animals such as transgenics:

14.2 Do the animals require housing other than standard caging/bedding or tanks/water?

- No  
 Yes (List housing required and explain here):

14.3 Will animals be housed singly?

- No  
 Yes (List reason single housing is required and explain why here):

Non-Human Primate Housing and Social Housing of Social Species criteria options can be found at: [http://grants.nih.gov/grants/olaw/positionstatement\\_guide.htm#nonhuman](http://grants.nih.gov/grants/olaw/positionstatement_guide.htm#nonhuman)  
[http://ursa.research.gsu.edu/files/2013/04/GSU\\_IACUC\\_SOP\\_on\\_Social\\_Housing\\_of\\_Social\\_Species.docx](http://ursa.research.gsu.edu/files/2013/04/GSU_IACUC_SOP_on_Social_Housing_of_Social_Species.docx)

14.4 Do the animals require special care?

- No  
 Yes (List special care and explain here):

14.5 Do the animals require diet other than the standard diet for this species used at GSU?

- No  
 Yes (List diet and explain here):

14.6 Will food and/or fluid restriction and/or regulations be necessary?

- No  
 Yes (List restrictions and explain here how they will be managed).

Describe how the animal(s) will be monitored to ensure that food and fluid intake meets their nutritional needs.

- 14.7 Will animals remain outside the [IACUC approved Animal Housing Area](#) for more than 12 hours?  
 No  
 Yes (List building and room number and explain here):
- 14.8 Will animals be transported outside of an IACUC approved Animal Housing Area (e.g. to your lab or off campus)?  
 Yes  
 I agree to adhere to the [GSU IACUC Animal Transportation Policy](#).  
 I request a deviation from the [GSU IACUC Animal Transportation Policy](#). Please state the deviation and provide a justification for the deviation here:  
 No
- 14.9 Will animals undergo experimental manipulations outside the IACUC approved Animal Housing Area?  
 Yes (List building and room number and explain here):  
 No
- 14.10 Can animals be provided environmental enrichment? Enrichment options can be found at: [Enrichment Options](#)  
 Yes (List enrichment requested here): [group housing](#), [Nylabones](#), [shelters](#)  
 No (Explain reason for lack of enrichment here):

**GEORGIA STATE UNIVERSITY  
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE  
Request for Amendment to an Approved Protocol  
(revised 05/09/2014)**

**Information for the protocol you wish to amend:**

**Protocol Number: A15003**

**Principal Investigator: Jeffrey S. Otis, PhD**

**Department: Kinesiology and Health**

**Protocol Title: Myocardial Infarction and Cardiac Regeneration**

**Animal Type and Quantity: rat, 80**

**Proposal Period:**

1. I wish to amend this protocol by making the following changes:

→ This amendment seeks approval for **four** additions to the parent protocol:

- (1) An additional 80 experimental animals
- (2) Use of resveratrol (RSV: 10mg/kg/day delivered i.p.) to be started on the day of surgery and continuing for 28 days of recovery
- (3) Ear punches
- (4) Minor protocol change that allows us to sacrifice animals in our lab (using approved techniques: CO<sub>2</sub> inhalation followed by thoracotomy to confirm death).

2. Please provide scientific justification for the changes. *(if you are **only** adding Genetically Modified Animals (GMAs), with their inherent potential for unanticipated phenotypes, please describe monitoring procedures for unexpected outcomes. If you are only adding GMAs and there are no new procedures you may proceed to Question 8).*

→ (1) **Animals:** The parent protocol was a training protocol intended to improve the surgical proficiency of Russell Rogers during the left anterior descending (LAD) artery ligation surgery. This training is still ongoing and we submit this amendment now to initiate the review process. In reality, these newly requested animals will not be used until later this summer when we (and the veterinary staff) are comfortable with his progress and outcomes.

Effect size, power, and required sample size to attain power of 0.8 at alpha level of 0.05 have been calculated. Including ~25% more animals/group to account for attrition or surgical complication, each experimental group will require 20 animals. Thus, we anticipate that 80 rats will be required and randomly assigned to 1 of 4 groups.

- a) Sham operated, vehicle (dimethyl sulfoxide, DMSO)
- b) Sham operated, RSV (10 mg/kg/day, i.p., diluted in DMSO)
- c) LAD artery ligation, vehicle (DMSO)
- d) LAD artery ligation, RSV (10 mg/kg/day, i.p., diluted in DMSO)

(2) **Resveratrol:** Resveratrol (RSV, 3, 5, 4'-trihydroxystilbene) is a powerful antioxidant that is increasingly being used to treat several common chronic diseases such as cancer, cardiovascular and pulmonary diseases. For example, resveratrol has been shown to provided cardioprotection, in part, by restoring Nrf2 activity (a central transcription factor that drives expression of several antioxidants), improving glutathione levels, and attenuating caspase 3 activity. We are currently defining the impact of resveratrol on signaling pathways known to regulate skeletal muscle repair and these initial data are encouraging and suggest that similar benefits may be achievable in the infarcted heart as well.

RSV (10 mg/kg/day for 28 days, delivered i.p.) will be dissolved in DMSO and sterile-filtered using 0.22- $\mu$ m syringe filters.

- (3) **Ear punches:** Ear punches will be performed under isoflurane sedation while the animal is undergoing the LAD artery ligation surgery. This, in combination with notation on cage cards and detailed surgical records in our log book, will ensure correct identification of our animals. The punch will be cleaned between each animal with alcohol.
- (4) **Euthanasia location:** The parent protocol was a training protocol and animals were not leaving the DAR facility. These newly requested experimental animals will be sacrificed in our lab and tissue collections will need to occur immediately. We will euthanize animals as approved using CO<sub>2</sub> inhalation followed by thoracotomy to confirm death. This location change will affect all animals.

3. Justification for non-duplication of work.

Please provide a written assurance that the proposed work is not unnecessarily duplicative.

→ We assure that this requested work does not unnecessarily duplicate previous findings.

4. Personnel: If new procedures are proposed, list the name(s), experience and/or training of the personnel and the procedures that each will conduct. List the names(s) of the individuals who will be responsible for training:

→ (1) Russell Rogers (PhD student): Russell will be responsible for all work described in this amendment and the parent protocol. Russell has 5 years of experience working with rodent models, including small animal handling, surgical techniques, and post-procedural care. He has recently been trained by DAR staff on i.p. injection.

(2) Jeffrey S. Otis (PI): Dr. Otis has over 15 years of experience working with mouse and rat models – including survival surgery models. He will oversee all aspects of this amendment.

5. Highest Pain Category for experiments described in this amendment: → (enter B, C, D, or E)  
C.

**USDA Classifications and Examples of Pain Categories:**

**Classification B:** Animals being bred, conditioned, or held for use in teaching, testing, experiments, research, or surgery, but not yet used for such purposes.

**Classification C:** Animals upon which teaching, research, experiments, or tests will be conducted involving no pain, distress, or use of pain-relieving drugs.

**Classification D:** Animals upon which experiments, teaching, research, surgery, or tests will be conducted involving accompanying pain or distress to the animals and for which appropriate anesthetic, analgesic, or tranquilizing drugs will be used.

**Classification E:** Animals upon which teaching, experiments, research, surgery, or tests will be conducted involving accompanying pain or distress to the animals and for which the use of appropriate anesthetic, analgesic, or tranquilizing drugs will adversely affect the procedures, results, or interpretation of the teaching, research, experiments, surgery, or tests.

Is this a change in Pain Category?  Yes  No

6. If the Pain Category is “B” or “C” please go to question number 8.

7. Explanation for the Consideration of Alternatives for Category “D” or “E” Animal Use.

Federal Regulations (The Public Health Service Policy and the [Animal Welfare Act](#)) and [University Policy](#) require assurance that this project does not unnecessarily duplicate research projects/courses performed at this or other institutions, and that the use of alternatives to live animal models and alternative procedures that may cause more than momentary or slight pain/distress (Class “D” and “E” procedures) to animals have been considered. The information in this section should include adequate information for the IACUC to assess that a reasonable and good faith effort was made to determine the availability of alternative models or methods.

**The following is a guide for answering 7.1 and beyond.**

**Your literature search is done as part of the OLAW requirement to address the “3Rs” (Refinement, Reduction, and Replacement) issues. For example:**

\* **Refinement** of procedures to eliminate or minimize pain or distress, the use of remote telemetry to decrease the distress of restraint; the use of humane endpoints.

Other examples of refinement are: ways to enhance the well-being of animals and the use of analgesics to decrease pain or anesthetics to decrease distress.

For the literature searches

**Ask "Am I using the least painful technique(s)?"**

- **Search terms:** Your species + animal/experimental model/technique + scientific keywords

\* **Replacement** of live animals with non-animal procedures or a less sentient species. Examples include the use of non-animal models such as in-vitro work, cell culture, tissues culture, computer models or simulations.

**Ask "Do I need to use animals?":**

- **Search terms:** Your experimental model + simulation + in vitro +scientific keywords

**Ask "Am I using the lowest species possible?":**

- **Search terms:** Your species + all applicable lower species (For example if using mice, specifically use "invertebrate", "fish" and "frog" in your search terms) + your animal/experimental model + scientific keywords

**Note: There are NO search terms that will direct the searcher to all examples of replacement or refinement. The onus is on the researcher to read and evaluate the literature.**

\***Reduction** in the number of animals used in the study. (**This only needs to be addressed in Question 14 below**). Examples include the use of shared control groups; preliminary screening in non-animal systems; innovative statistical packages.

***The selection of databases depends on the work performed and the species used in the protocol. Samples of databases available through the Georgia State University Library [www.library.gsu.edu/database](http://www.library.gsu.edu/database) web pages***

· *PubMed* · *Web of Knowledge* · *Biological Abstracts* · *Web of Science* · *PsychINFO* · *Alt-Web* · *NLM Gateway* · *AVAR* · *Galileo* · *AGRICOLA*

*OID (allows a search of multiple databases including Agricola, BIOSIS, CAB Abstracts, Medline, Zoological Record, etc.)*

Please visit the Animal Welfare Information Center (**AWIC**) Literature Searching and Databases page ([http://awic.nal.usda.gov/nal\\_display/index.php?tax\\_level=1&info\\_center=3&tax\\_subject=184](http://awic.nal.usda.gov/nal_display/index.php?tax_level=1&info_center=3&tax_subject=184)) for a list of databases that can be used to search for alternatives. The AWIC site also recommends using the Literature Search Worksheet (<http://www.nal.usda.gov/awic/alternatives/searches/altwksht.pdf>) to assist in performing a successful alternatives database search. The worksheet helps to identify relevant searchable terms and concepts.

### 7.1 Literature search for alternatives to painful procedures

If you chose category “D” or “E” above, please do literature searches using the broadest database for your area of study, and provide a brief summary of the results obtained to verify that you investigated the use of alternatives to painful or distressful procedures. If you have not selected either category D or E, skip to section 6.2.



7.1.1 Name of the databases used: →

Date the databases search(es) were done: →

Did the search cover the entire date range of the databases with no restriction on dates?

Yes

No (If No, provide dates covered by the search(es) below)

Dates covered by the databases search(es): →

7.1.2 Search keyword(s) used: include number of hits for combinations of terms. **You must use ‘alternative’ and ‘animal welfare’ in the search combinations for invasive or painful procedures** (See above for 3R criteria.)



7.1.3 Summarize the information found in the hits below. (Note: this should be a general summary. You do not need to go into the details of each hit but rather summarize hits by relevant groups)



7.1.4 Explain what alternatives exist in place of using animals for this protocol and provide a justification if these cannot be used.



#### 7.1.5 Other Sources of Information on Alternatives to Painful Procedures

7.1.5.1 Consultation with Experts: (Names, credentials, and dates): →

7.1.5.2 Scientific Meetings: Specify: →

*Note: A careful literature search is usually the best way to determine whether a proposed study is unnecessarily duplicative of previous work. However, it is ok to repeat a published experiment to make sure that it works in a different lab. This reason just needs to be stated as the justification.*

8. If additional animals are needed, please justify their use.

Note: This Amendment Form may be used to request additional animals. If a different species is required you must submit a new protocol.

→ Animal use justification has been provided in question 2.

9. Are there any changes in the Animal housing/husbandry for this protocol?  
If so please describe below

→ No.

**Jeffrey S. Otis** \_\_\_\_\_  
Investigator's Signature

\_\_\_\_\_  
Date

PLEASE RETURN THIS FORM TO:  
IACUC Compliance Officer / Georgia State University / Office of Research Integrity / 232 Alumni  
Hall / Atlanta, GA 30303 e-mail: iacuc@gsu.edu

**APPROVED:** \_\_\_\_\_  
Attending Veterinarian's Signature

\_\_\_\_\_  
Date

**APPROVED:** \_\_\_\_\_  
IACUC Chair's Signature

\_\_\_\_\_  
Date